Role of Internal Lysine Residues of Serum Albumins in Bilirubin Binding and Conformational Stability of Albumin-Bound Bilirubin

Abstract

Mohammad Mushahid Khan

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in Biotechnology of the Aligarh Muslim University
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ABSTRACT

Bilirubin, a bichromophoric tetrapyrrole, binds to albumin with high affinity at a site located at or near loop 4 in subdomain IIA. Bilirubin-albumin binding is stabilized by various noncovalent forces such as salt linkages, hydrogen bonds, aromatic $\pi-\pi$ van der Waals attractions and hydrophobic interactions. The combination and topography of these binding elements dictate the three-dimensional shape and chirality of the bound pigment. In terms of the relative importance, salt linkages existing between carboxyl groups of bilirubin and ε-NH_2 groups of lysine residues in protein, are presumed to be more important. These lysine residues are believed to be buried in the protein interior having abnormal pK values. Although, involvement of internal lysine residues in bilirubin-albumin interaction has been shown in BSA, a detailed and careful investigation is lacking on other serum albumins. In order to understand the mechanism of bilirubin encephalopathy and to develop various preventive measures, selection of an animal is futile unless the animal albumin binds bilirubin in the similar manner as human albumin binds. In view of this, different mammalian serum albumins viz. human (HSA), rabbit (RbSA), goat (GSA), sheep (SSA) and buffalo (BuSA) serum albumins were selected and their conformational and bilirubin binding properties were studied after modifying the internal lysine residues with acetic anhydride, succinic anhydride and O-methylisourea.

In order to modify the internal lysine residues, first the reactivity of completely or partially exposed lysine residues of these albumins, was checked towards citraconic anhydride, a reversible modifier. A progressive increase in citraconylation was observed with the increase in citraconic anhydride concentration up to ~100 molar excess of anhydride over protein which sloped off at higher anhydride concentration. A comparison of these results suggested that in all albumins, nearly 80±3% lysine residues were completely accessible to the modifier whereas remaining 20±3% were buried to different extent in the protein interior and thus required higher anhydride concentration for their modification. These lysine residues were modified using a double chemical modification approach as described in Materials and Methods. All these derivatives (acetylated, succinylated and guanidinated), having ~10-13 internal lysine residues modified, were found to be homogeneous with respect to charge and size as judged by Seralose-6B gel chromatography and polyacrylamide gel electrophoresis. However, a slight increase in the anodic mobility was observed in some of the succinylated albumin derivatives. These derivatives were then characterized for their conformational and bilirubin binding properties using analytical gel filtration, circular dichroism (CD), fluorescence and absorption spectroscopy.

As characterized by far-UVCD (MRE_{222nm}), no significant change was observed in the secondary structure of these albumins upon covalent modification of internal lysine residues. On the other hand, near-UVCD spectra of acetylated as well as succinylated derivatives showed a slight decrease in the MRE value both at 262 and 268 nm and a

little increase in MRE above 280 nm. These alterations in the MRE were indicative of the change in the asymmetry of the protein's aromatic amino acids' environment and / or disulfide bridges. The near-UVCD spectra of guanidinated albumin derivatives were more or less similar to the one obtained with native albumins. A slight increase in the ellipticity value between 280 and 300 nm in acetylated and succinylated derivatives can be ascribed to the immobilization of tryptophan side chain in a more hydrophobic environment which was in accordance with the tryptophan fluorescence results in which a blue shift of about 9-11 nm in the emission maxima was observed in these derivatives. These results suggested that few acetyl or succinyl groups were incorporated on to those lysine residues which seem to be located in the close vicinity of tryptophan. Since the extent of blue shift and decrease in fluorescence intensity observed with HSA (containing a lone tryptophan-214 in subdomain IIA) upon acetylation or succinylation were more or less similar to those observed with the same derivatives of other albumins, it was believed that change in the microenvironment was mainly confined to the tryptophan present in subdomain IIA in these albumins. Although, all serum albumins showed an increase (5-8%) in Stokes radius upon succinylation, the effect of acetylation on the Stokes radius was seen only in some cases. Guanidination, on the other hand, did not result in any change in Stokes radius. Taken together, all these results suggested a slight alteration in the tertiary packing of different albumins upon acetylation and succinylation. Guanidination did not affect the conformation of protein to a measurable extent.

Using fluorescence quench titration for the interaction of bilirubin with albumin and by fitting the data in Scatchard plot, a significant decrease (5-21 fold) in the value of association constant, K_a was noticed upon acetylation or succinylation of internal lysine residues in different serum albumins. On the other hand, guanidinated albumin derivatives showed more or less similar values of K_a as obtained with native albumins. Visible absorption spectra of bilirubin-albumin complexes were severely perturbed upon acetylation or succinylation. Nearly 20-30 % reduction in molar absorption coefficient (ε) and a significant blue shift (10-20 nm) in the absorption maxima (λ_{max}) of various bilirubin-albumin complexes were noticed upon acetylation or succinylation of internal lysine residues of these albumins. On the other hand, guanidination showed little changes in these properties. The intensity of both positive and negative CD Cotton effects (CDCEs) of the characteristic bisignate CD spectra of bilirubin-HSA and bilirubin-RbSA complexes was reduced to ~50% to that of the original value upon acetylation or succinylation of internal lysine residues, though the shape and sign order of the bisignate CD spectra were identical to that observed with native albumins. Interestingly, characteristic monosignate CD spectra of bilirubin complexed with GSA, SSA and BuSA, were transformed to the bisignate CD spectra upon acetylation or succinylation of internal lysine residues. Guanidination, on the other hand, did not perturb the CD spectra of bilirubin-albumin complexes. These results suggested the predominance of salt linkage(s) contributed by internal lysine residue(s) in binding and dictating the three dimensional shape and chirality of bilirubin at high affinity site on

different albumins. When probed by CD spectroscopy, bilirubin bound to acetylated or succinylated derivatives of GSA and SSA was rapidly switched over to native albumins but not vice versa, implying that salt linkage(s) provided stability to the native three dimensional conformation of the bound pigment. Chloroform efficiently transformed the monosignate CDCEs of bilirubin complexed with GSA, SSA and BuSA into bisignate CDCEs and inverted the bisignate CDCEs of bilirubin complexed with HSA and RbSA. Chloroform also severely decreased the intensity of both positive and negative CDCEs of bilirubin complexed with acetylated or succinylated derivatives of all albumins which otherwise increased significantly in case of native as well as guanidinated albumin-bilirubin complexes except bilirubin-RbSA complex where a little decrease in intensity was found. Further, except HSA derivatives, chloroform did not invert the CD spectra of bilirubin complexed with acetylated or succinylated derivatives of other albumins. Alterations in the CD spectra of bilirubin complexed with guanidinated derivatives were similar to native albumin-bilirubin complexes, thus the existence of salt linkage(s) in bilirubin-albumin complexation seems to be crucial to bring about effective and efficient stereochemical changes of the bound pigment by co-binding of chloroform which seems to have at least one conserved binding site on different serum albumins that is shared with bilirubin.

Photoinduced fluorescence enhancement of bilirubin bound to primary binding site on HSA was completely ceased upon acetylation or succinylation of internal lysine residues, though the pigment bound to these derivatives in a folded conformation as

revealed by retention of bisignate CD spectra, akin to that bound to HSA. Interestingly, when characterized by CD spectroscopy, a brief (1 minute) irradiation of bilirubin bound to acetylated or succinylated HSA derivatives under white fluorescent light, completely abolished the negative CDCEs while moderately affecting the intensity of positive CDCEs and shifting the band maxima slightly towards blue region. On the other hand, 1 minute irradiation of bilirubin bound to native or guanidinated HSA under white fluorescent light, produced only a little decrease in the intensity of both positive and negative CDCEs. Similar changes were also noticed in the visible absorption spectra of bilirubin complexed with HSA and its derivatives. A brief (2 minutes) irradiation of an equimolar complex of bilirubin with either acetylated or succinylated HSA accompanied a rapid shift of ~15 nm in the absorption maxima (λ_{max}) towards shorter wavelength which continued up to 4-5 minutes and then became constant at 434/ 435 nm. On the other hand, similar treatment of the complexes of bilirubin with either native or guanidinated HSA derivative did not show any change in the absorption spectra.

These photoinduced spectral modulations can not be ascribed to the binding of bilirubin to secondary low affinity sites as the CD spectrum of bilirubin bound to these derivatives showed complete inversion upon addition of chloroform which binds to subdomain IIA in HSA where high affinity bilirubin binding site is located. Further, presence of chloroform reconciled the photoinduced alterations in the CD spectrum observed in its absence, suggesting that chloroform stabilized the bound ligand against

light but the fluorescence properties of bilirubin complexed with acetylated or succinylated derivatives remained unchanged. Guanidination of internal ε-NH_2 groups in HSA by O-methylisourea did not alter the spectral properties of the bound ligand. These results suggest that salt linkage(s) existing between ε-NH_2 groups of lysine residues in HSA and carboxyl groups of bilirubin, act(s) as a potential barrier during conformational rotation of the bound ligand assisted by photoactivation and their abolishment can alter the dynamics and stereoselectivity of the bound pigment, a hitherto unnoticed implication of salt linkage(s) in bilirubin-HSA complex.

ROLE OF INTERNAL LYSINE RESIDUES OF SERUM ALBUMINS IN BILIRUBIN BINDING AND CONFORMATIONAL STABILITY OF ALBUMIN-BOUND BILIRUBIN

MOHAMMAD MUSHAHID KHAN

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in Biotechnology of the Aligarh Muslim University, Aligarh

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Certificate

I certify that the work presented in the following pages has been carried out by Mr. Mohammad Mushahid Khan and that it is suitable for the award of Ph.D. degree in Biotechnology of the Aligarh Muslim University, Aligarh.

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Declaration

I hereby declare that the thesis entitled "Role of Internal Lysine Residues of Serum Albumins in Bilirubin Binding and Conformational Stability of Albumin-Bound Bilirubin", embodies the work carried out by me.

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(MOHAMMAD MUSHAHID KHAN)

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LIST OF ABBREVIATIONS AND SYMBOLS USED

CD	:	Circular dichroism
CDCE	:	CD Cotton effect
MRE	:	Mean residue ellipticity
TNBS	:	Trinitrobenzene sulfonic acid
PAGE	:	Polyacrylamide gel electrophoresis
Tris	:	Tris [hydroxymethyl] aminomethane
TEMED	:	N, N, N', N'-tetramethylethylenediamine
EDTA	:	Ethylenediaminetetraacetic acid
ε-NH_2 groups	:	Epsilon amino groups
BSA	:	Bovine serum albumin
BuSA	:	Buffalo serum albumin
aBuSA	:	Acetylated buffalo serum albumin
gBuSA	:	Guanidinated buffalo serum albumin
sBuSA	:	Succinylated buffalo serum albumin
GSA	:	Goat serum albumin
aGSA	:	Acetylated goat serum albumin
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HSA	:	Human serum albumin
aHSA	:	Acetylated human serum albumin
gHSA	:	Guanidinated human serum albumin
sHSA	:	Succinylated human serum albumin
RbSA	:	Rabbit serum albumin
aRbSA	:	Acetylated rabbit serum albumin
gRbSA	:	Guanidinated rabbit serum albumin
sRbSA	:	Succinylated rabbit serum albumin
SSA	:	Sheep serum albumin
aSSA	:	Acetylated sheep serum albumin
gSSA	:	Guanidinated sheep serum albumin
sSSA	:	Succinylated sheep serum albumin
ϵ	:	Molar absorption coefficient
$[\theta]$	:	Molar ellipticity
K_a	:	Association constant

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Introduction

INTRODUCTION

Serum albumin is one of the members of a multigene family of proteins that includes α-fetoprotein (AFP) and human group-specific component (Gc) or Vitamin D-binding protein (Carter & Ho, 1994). It is the major soluble protein constituent of the circulatory system having many vital physiological functions and is chiefly responsible for the maintenance of blood pH. It also contributes ~80% to the colloid osmotic blood pressure (Figge *et al.*,1991). Serum albumin was recognized as a principal component of blood as early as 1839 (Ancell, 1839). Originally, the protein was referred to as 'albumen', derived from the Latin word *albus* 'meaning white' after the white color of flocculant precipitates produced by various proteins. Because of its availability, low cost, stability and unusual ligand binding properties, serum albumin has been one of the most exhaustively studied and applied proteins in biochemistry as evident from several excellent reviews (Foster, 1960; Peters, 1970; 1980; 1984; 1985; 1992; Kragh-Hansen, 1981; 1990; Carter & Ho, 1994) and a book (Peters, 1996) on serum albumin.

Biosynthesis

The synthesis of albumin takes place in the liver as has been demonstrated in surviving slices of liver from many species (Peters & Anfinsen, 1950; Dancis *et al.*, 1957; Hirayama *et al.*, 1959). A systematic analysis of the synthesis of nascent polypeptide chain, disulfide bonds formation and its delivery into endoplasmic reticulum and finally to

other destinations has been made by Peters and his group (Peters, 1962a; 1962b; Peters & Peters, 1972; Peters & Reed, 1980; Peters & Davidson, 1982). In fetus, the synthesis of albumin starts by about 7 weeks (Kelleher *et al.*, 1963; Gitlin & Boesman, 1966) and in early fetal circulation there is another protein, α_1-fetoglobulin, of molecular weight 65,000 which differs from albumin in amino acid composition and electrophoretic mobility (Bergstrand & Czar, 1956; Alpert *et al.*, 1968). Albumin mRNA is comprised of 2080 base pairs. Signal peptide, a stretch of 18 residues, is the first translated sequence which directs the growing peptide chain through the membrane of endoplasmic reticulum before being cleaved off and even before the completion of the RNA translation. The newly formed albumin called 'proalbumin' contains a stretch of a basic hexapeptide (Arg-Gly-Val-Phe-Arg-Arg) attached to its amino terminal end (Urban *et al.*, 1974). This leader peptide guides the polypeptide chain from endoplasmic reticulum to Golgi complex for proteolytic processing and secretion (Judah, 1983) and finally cleaved in the Golgi complex (secretory vesicles) before the release of albumin from the cell in its biologically active form.

Distribution and turnover

Serum albumin is located in every tissue and bodily secretions. About 40% of the total albumin is found in the circulatory plasma (Peters, 1992) whereas remaining 60% resides about half in viscera and half in muscle and skin (Rabilloud *et al.*, 1988). Albumin is a major protein of tooth "enamelin" (Strawich & Glimcher, 1990) and is reported to occur

intracellularly in developing brain tissue (Pineiro *et al.*, 1982), nerve cells (Mata *et al.*, 1987) and both interstitially and intracellularly, in testis (Krishna & Spanel-Borowski, 1990) where it may be produced as "testibumin" in the seminiferous tubular compartment (Cheng & Bardin, 1986). Albumin has also been recognized in milk (Phillippy & McCarthy, 1979), amniotic fluid (Bala *et al.*, 1987) and mammary cysts (Balbin *et al.*, 1991). The albumin concentration in plasma in an average person is about 35-50 g/L, which declines slightly with age (Cooper & Gardner, 1989) and is lower in newborns (Cartlidge & Rutter, 1986) and as low as 20g/L in premature infants (Reading *et al.*, 1990). Albumin is produced by the liver at a rate of 0.7 mg / g liver per hour (Peters, 1985). About 4-5% of albumin is replaced by hepatic synthesis daily (Olufemi *et al.*, 1990). The turnover is first order with an average half-life of 19 days (Waldmann, 1977). The rate of albumin degradation generally falls in malnourished states including diabetes (Peters, 1992). The albumin concentration in plasma is maintained through transcriptional control of the albumin gene by the anabolic hormones, insulin and somatotropin (Hutson *et al.*, 1987). Albumin synthesis is also highly dependent upon the supply of dietary amino acids (Kaysen *et al.*, 1989).

Structural organization

Amino acid composition –Table 1 shows the amino acid composition of human (HSA), bovine (BSA), rat (RSA) and sheep (SSA) serum albumins. Albumins do not contain any carbohydrate or any other non-protein moiety, characterized by a low content of

Table 1

Amino acid compositions of human, bovine, rat and sheep serum albumins

Amino acid	*Human*[a]	*Bovine*[a]	*Rat*[b]	*Sheep*[b]
Aspartic acid	36	40	33	44
Asparagine	17	14	20	14
Threonine	28	33	34	30
Serine	24	28	24	24
Glutamic acid	62	59	57	56
Glutamine	20	20	23	19
Proline	24	28	29	29
Glycine	12	16	18	17
Alanine	62	47	61	51
Cystine /2	34	34	34	34
Cysteine	01	01	01	01
Valine	41	36	35	36
Methionine	06	04	06	04
Isoleucine	08	14	15	13
Leucine	61	61	56	62
Tyrosine	18	20	19	20
Phenylalanine	31	27	26	27
Histidine	16	17	15	18
Lysine	59	59	53	60
Tryptophan	01	02	01	02
Arginine	24	23	24	22
Total	585	583	584	583

[a]Peters, 1992; [b]Carter & Ho, 1994

isoleucine, methionine and glycine and contain either one (e.g. HSA and RSA) or two (e.g. BSA and SSA) tryptophan residues (Carter & Ho, 1994). Albumins have high content of cystine and charged amino acids, aspartic and glutamic acids, lysine and arginine (see Table 1) (Brown & Shockley, 1982). There is a tendency towards higher isoleucine and methionine content with lower species (Peters, 1992).

Primary structure – Mammalian albumins contain ~585 amino acids arranged in a single large polypeptide chain (Brown, 1975). Figure 1 shows the primary amino acid sequence of HSA. High percentages of sequence homologies have been found among different albumins being 76% between HSA and BSA (Peters, 1985); 76% between ESA (equine serum albumin) and HSA; 73% between ESA and BSA; 76% between ESA and RSA (Ho *et al.*, 1993) and 61% among BSA, RSA and HSA (He & Carter, 1992). About 50% conserved residues are found in all known albumin sequences (Carter & Ho, 1994). There are 17 disulfide bridges in albumin and the whole molecule is characterized by a unique arrangement of double loops that repeat as a series of triplets. They are arranged in a series of nine loop-link-loop structures centered around eight sequential Cys-Cys pairs (Figure 1). The conformations of the disulfides are primarily gauche-gauche-gauche ($C\beta_1$-S_1-S_2-$C\beta_2$) with torsion angles clustering around $\pm 80^{\circ}$ (He & Carter, 1992). The repetition of these loops in a triplet fashion of large-small-large can group these loops into three homologous domains (I, II & III) comprising of residues 1-190, 191-382 and 383-

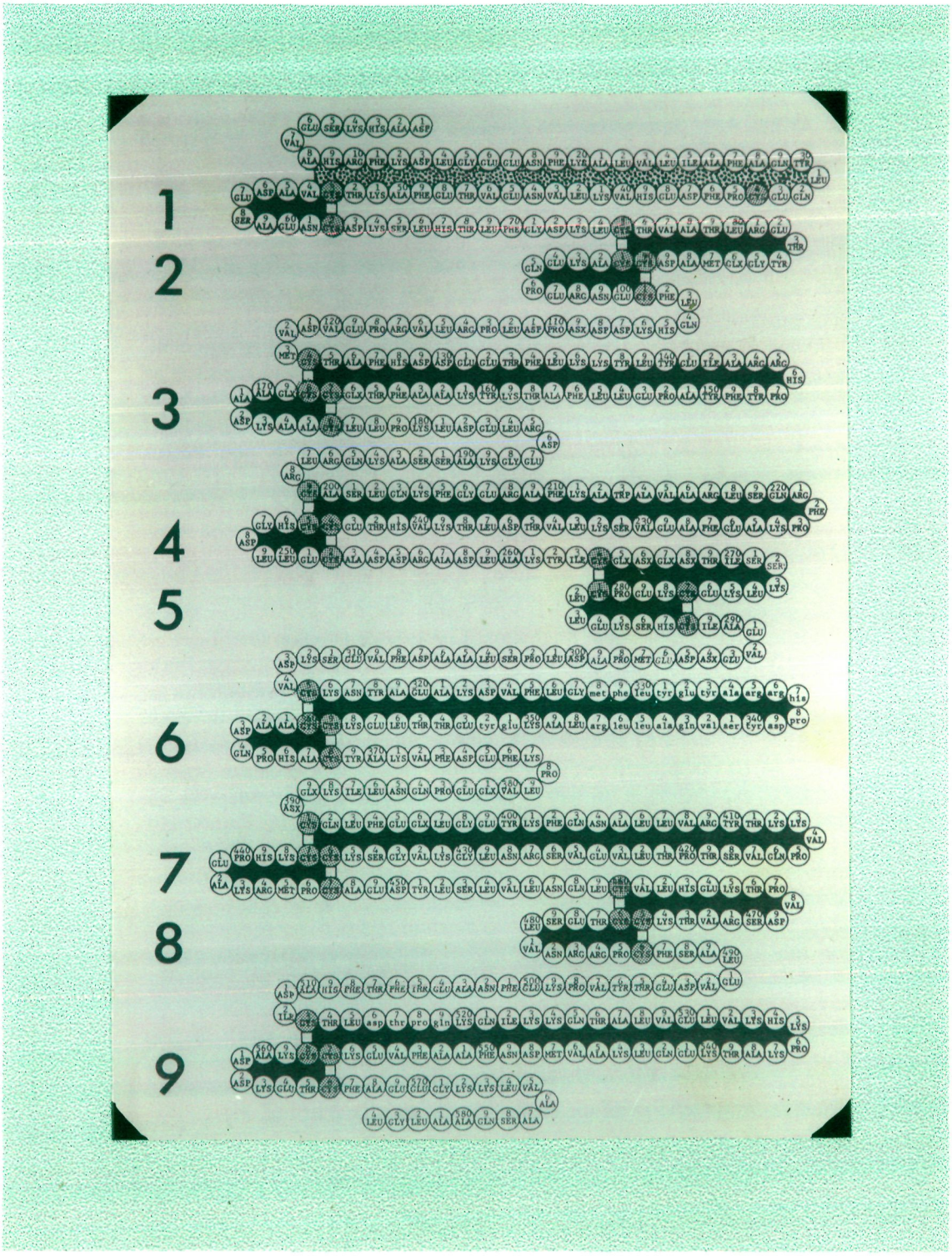


Figure 1. Illustration of the primary structure of human serum albumin (Adapted from Peters, 1985)

585 in the primary sequence of albumin (Brown, 1977). The sequence homology between these domains is 18-25% (Brown, 1977). The charge distribution of HSA at neutral pH for domains I, II and III are -9, -8 and +2 respectively (Peters, 1985). From the observations of loops and other homologies, Brown (1977) and McLachlan & Walker (1977) deduced that albumin has evolved from smaller proteins, one third or even one ninth of the present size by gene fusion.

X-ray structure–The three dimensional structure of HSA has been determined by X-ray crystallography at a resolution of 2.8 Å by He & Carter (1992) and more recently at 2.5Å resolution by Sugio and his group (Sugio *et al.*, 1999). Each of the three homologous domains (I, II & III) of albumin is further divided into two subdomains 'A' and 'B' respectively and these domains are arranged in such a way to give rise a heart shaped molecule (Figure 2) that can be approximated to an equilateral triangle with sides of 80 Å and depth of ~30 Å (He & Carter, 1992). The subdomains are extensively cross-linked by disulfide bridges which form the basis of the occurrence of 9 loops (3 in each domain) in albumin molecule. Albumin structure is predominantly α-helical (Jacobsen, 1972; Sjoholm & Ljungstedt, 1973; Chen & Lord, 1976; McLachlan & Walker, 1977). There are 10 principal helices in each domain, h1-h6 for subdomain 'A' and h7-h10 for subdomain 'B'. Further, the six subdomains share a common helical motif which corresponds to the amino acids encompassed within the double disulfide loops 1, 3, 4, 6, 7

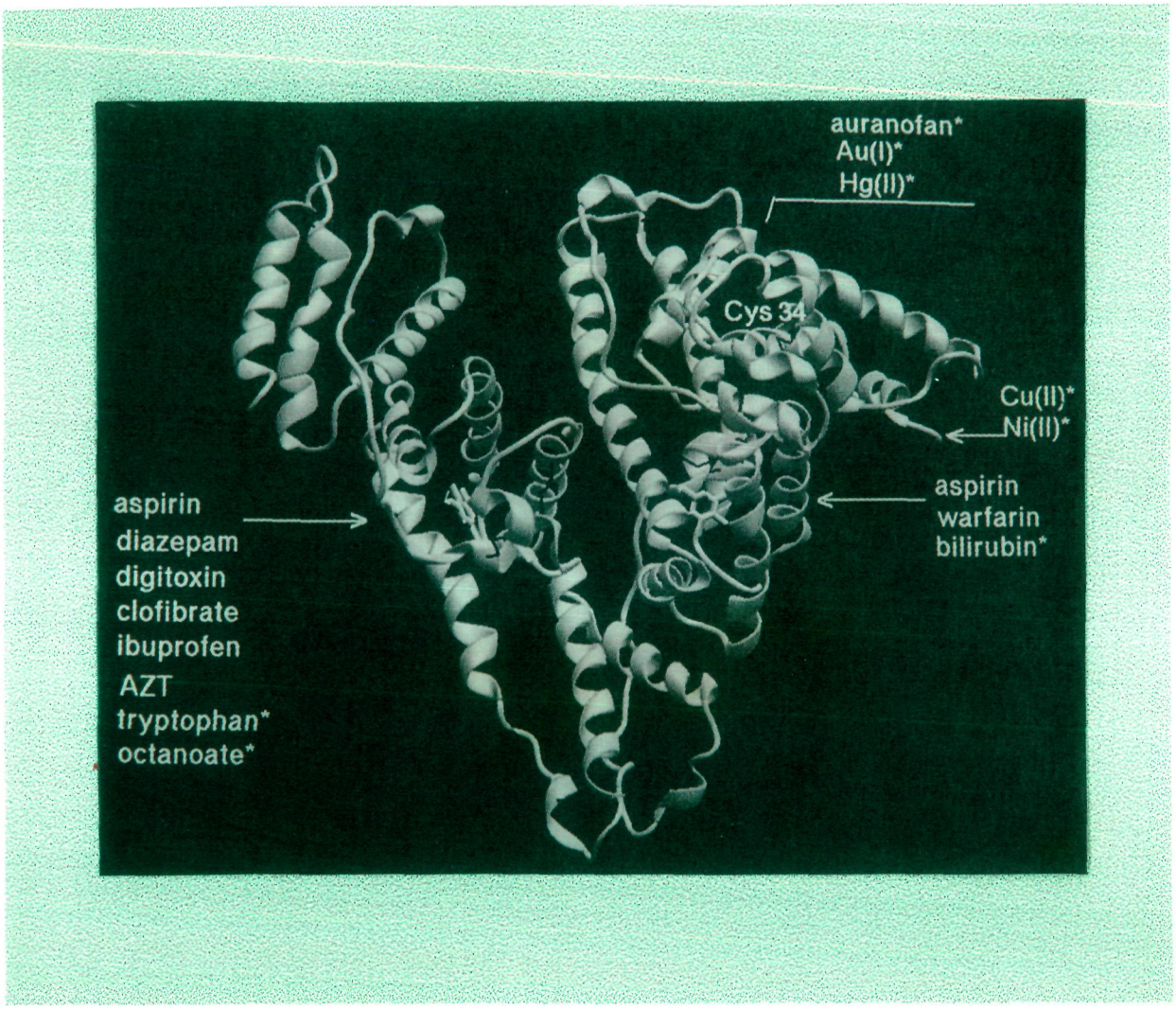


Figure 2. Stereo view of the heart shaped structure of human serum albumin with overall topology and secondary structure (Adapted from Carter & Ho, 1994)

and 9 wherein each motif is related by a pseudo twofold axis (I:168°, II:163°, III:171°) (Carter & Ho, 1994). Distinct differences between subdomains 'A' and 'B' also exist. The 'A' subdomains supplement the three-helix bundle on the C-terminal side with an additional but smaller disulfide double loop (loops 2, 5, and 8) to form a small globin-like fold that is extensively cross-linked by four disulfide bridges. The 'B' subdomains supplement the helical motif on the N-terminal side with a conformationally extended polypeptide to create a folding topology that closely resembles a simple up-down helical bundle. subdomains 'A' and 'B' assemble through hydrophobic helix packing interactions primarily involving h2, h3 and h8. Additionally, the subdomains are linked together by a presumed flexible extension of polypeptide encompassing residues Lys_{106}—Glu_{119}, Glu_{292}—Val_{315} and Glu_{492}—Ala_{511} in the domains I, II and III respectively. Domains I-II and II-III in turn are connected through extensions of h10(I) - h1(II) and h10(II) - h1(III) respectively creating the two longest helices in HSA. The actual number of helices in HSA is therefore 28 rather than 30 (Carter & Ho, 1994).

Physico-chemical properties

Physico-chemical properties of HSA and BSA are summarized in Table 2. The molecular weight of albumins is less than that of most of the other plasma proteins and ranges in between 66,000 and 67,000 (Loo *et al.*, 1991). Under neutral conditions of pH, albumin has an axial ratio of approximately 2.66 based on the X-ray crystallographic data which is in agreement with the value of 3.0 obtained from the studies of dielectric and

Table 2

Physico- chemical properties of human and bovine serum albumins

Properties	*Human*	*Bovine*
Molecular weight	66,248[a]	66,210[a]
Sedimentation constant, $S_{20,w} \times 10^{13}$	4.6[b]	4.5[c]
Diffusion constant, $D_{20,w} \times 10^{7}$	6.1[b]	5.9[d]
Partial specific volume, V_{20}	0.733[b]	0.733[e]
Intrinsic viscosity, η	0.042[b]	0.0413[f]
Frictional ratio, f/f_0	1.28[b]	1.30[g]
Overall dimensions, Å	38x150[h]	40x140[c]
Isoelectric point	4.7[h]	4.7[i]
Optical absorbance, $A^{1gm/liter}$ at 279nm	0.531[j]	0.667[j]
Mean residue rotation, $[m']_{233}$	8590[k]	8443[l]
Estimated		
α-helix, %	65[m]	54[n]
β-structure, %	15[o]	18[p]

[a]Peters (1985), [b]Oncley *et al.* (1947), [c]Squire *et al.* (1968), [d]Wagner & Scheraga (1956), [e]Hunter (1966), [f]Loeb & Scheraga (1956), [g]Creeth (1952), [h]Hughes (1954), [i]Longsworth & Jacobsen (1949), [j]Cohn *et al.* (1947), [k] Wallevik (1973), [l]Moore & Foster (1968), [m]Dockal *et al.* (2000), [n]Sogami & Foster (1968), [o]Sjoholm & Ljungstedt (1973), [p]Reed *et al.* (1975).

birefringence relation times (Moser *et al.*, 1966). The solvent-accessible surface and molecular volume of HSA are 28,202 $Å^2$ and 88,249 $Å^3$ respectively, calculated using the molecular coordinates and the algorithm of Richards (1985). The radius of gyration of HSA (26.7Å) is similar to the value (26.4Å) of hydrodynamic radius of HSA (rotational) measured by light scattering and electron spin resonance (ESR) (Cannistraro & Sacchetti, 1986). Hydrodynamically, HSA shows a sedimentation coefficient, $S^{o}_{20,w}$ of 4.5×10^{-13} S, diffusion coefficient, $D^{o}_{20,w}$ of 6.0×10^{-7} cm^2/sec (Oncley *et al.*, 1947) and partial specific volume of 0.733 cc/g (Charlwood, 1961). The intrinsic viscosity of the protein has been measured as 4.2 cc/g (Charlwood, 1961). Defatted albumin has an isoelectric point of ~5.3 whereas a value of ~4.7 has been found with fatted HSA. At physiological pH, the calculated net charge is 15 (Peters, 1985). High charge on the protein molecule is responsible for its high solubility in water and a solution as concentrated as 30% (w/v) can be prepared (Peters, 1985). The specific absorption coefficient, $E^{1\%}_{1cm}$ at 279 nm has been found to be 5.31 and 6.67 for HSA and BSA respectively (Edwards *et al.*, 1969; Janatova *et al.*, 1968; Peters, 1985).

Isomerization

Luetscher in 1939 first showed major reversible conformational isomerization of albumin with changes in pH (Luetscher, 1939). Foster studied these earlier observations in much greater detail (Foster, 1960; 1977) and classified the pH dependent forms as 'N', for

normal form, which is predominant at neutral pH; 'B', for *basic form* occurring above pH 8.0; 'F', for *fast migrating form* at pH values less than 4.0; 'E', for *expanded form* at pH less than 3.5 and 'A', for *aged form* existing with time at pH values greater than 8.0. Most of these isomerizations have been studied with BSA, but they apparently occur in a similar fashion with HSA.

The 'F' form was recognized by Foster in 1957 who demonstrated that the abrupt discontinuity in the titration of BSA with HCl where about 40 carboxyl side chains become titrable (Tanford, 1952) at pH 4.0-4.5 coincided with the appearance of a *faster migrating form* or 'F' form, as seen on gel electrophoresis at pH 3.0-4.0 (Aoki & Foster, 1957). Hydrodynamic properties such as sedimentation rate, diffusion constant and intrinsic viscosity show that the 'F' form is longer and increasingly asymmetrical (Carter & Ho, 1994). The 'F' form is characterized by a small (6-7%) decrease in the α-helical content and a significant (~40%) increase in β-sheet structure (Dockal *et al.*, 2000). Ultrasonic absorption and velocity spectra (Choi *et al.*, 1990) exhibited a relaxation frequency near 200 KHz attributed to the expansion of the molecule. In addition to the availability of 40 carboxyl groups to titration, 3 to 5 tyrosyl side chains become unmasked (Herskovits & Laskowski, 1962; Steinhardt *et al.*, 1971). There is a loss of about 12 binding sites for SDS (Foster, 1960) and a 40-50% increase in ANS binding (Foster, 1960).

At pH values below 3.5, a further unfolding occurs until about pH 2.5, leading the

molecule to an expanded form constrained by its disulfide bonds. This was termed as 'E', or *expanded form* by Foster (Foster, 1960). Acid expansion is fully reversible. At pH 1.7, the intrinsic viscosity increases markedly indicating a change in molecular size as axial ratio also increases from 4.0 to 9.0 (Harrington *et al.*, 1956). The molecule appears like a chain of globules about 21 x 250Å in size as seen under electron microscope (Slayter, 1965). The helical content decreases from 45% to 35% (Peters, 1985).

At pH 9.0, albumin undergoes another conformational change to a basic 'B' form. Based on UV difference spectral results at 287 nm, albumin shows increased exposure of some tyrosine residues (Williams & Foster, 1959) in the pH range 7.0 to 9.0. Tryptophan fluorescence and rotatory relaxation time exhibit little change (Peters, 1996). Small reduction (10%) in α-helical content and gain (8%) in β-structure has been found using CD data (Era *et al.*, 1990). The fluorescent dye ANS shows a decrease in rotatory relaxation time from 131 to 114 nanoseconds (Era *et al.*, 1990).

'A' form or *aged form* is formed when the albumin is left at pH 9.0 for 3-4 days in salt free solution (Sogami *et al.*, 1969). The 'A' form is characterized by a slower electrophoresis migration rate and by decreased solubility in 3 M KCl (Nikkel & Foster, 1971). It has been shown to be the result of a sulfhydryl catalyzed disulfide interchange reaction (Era *et al.*, 1990). Blocking of the thiol group with iodoacetamide prevents the formation of 'A' form as seen under electron microscope (Slayter, 1965). The helical content decreases from 45% to 35% (Peters, 1985).

Denaturation

The denaturation of serum albumin is reversible (Wallevik, 1973; Aoki *et al.*, 1974; Reisler *et al.*, 1977) as indicated by the capacity of refolded protein to bind specific antibodies, bilirubin and palmitate (Teale & Benjamin, 1977; Wichman *et al.*, 1977; Chavez & Benjamin, 1978; Johanson *et al.*, 1981). Albumin can withstand in 8 M urea or 6 M GdnHCl even at 44^{0}C with temporary loss of α-helix without any obvious harm (Tanford, 1968). However, prolonged or repeated heating or exposure to alkali causes dimerization, unfolding and eventual aggregation. Polymerization and loss of helix occurs in less than 1 minute when the protein is exposed to 65 ^{0}C at pH 9.0 (Aoki *et al.*, 1973). Fatty acid free albumin tends to aggregate more promptly than fatted albumin (Brandt & Andersson, 1976; Shrake *et al.*, 1984). A loss of α-helix (from 61% to 44%) and a gain of β-structure (from 6% to 16%) occur between 62°C and 75°C as judged by CD, infrared and laser Raman studies. The availability of S-S bonds increases from 5% at 60 ^{0}C to 47% at 100 ^{0}C (Peters, 1996). The protein is sensitive to the time of exposure at high temperature. Effects of heat up to 45°C (Takeda *et al.*, 1989) or to 20% of maximal denaturation (Wetzel *et al.*, 1980) are fully reversible whereas 60% reversibility is found after exposure to 80°C. Little changes occur in albumin molecule below 4 M urea concentration (Khan *et al.*, 1987) or 1.8 M GdnHCl (Wallevik, 1973; Katz *et al.*, 1973; Aoki *et al.*, 1974) followed by stepwise changes up to about 8 M urea which include a rapid initial change followed by molecular expansion (Chmelik *et al.*, 1988). Urea-

induced denaturation of defatted albumin proceeds via an intermediate formation where major conformational changes occur in domain III (Khan *et al.* 1987; Muzammil *et al.*, 2000).

Functions of albumin

Albumin performs many vital functions in the body while circulating in blood plasma. It is mainly responsible for the maintenance of blood pH and contributes ~80% to the colloid osmotic blood pressure. It is the chief reservoir and carrier for long chain fatty acids (Brodersen *et al.*, 1991; Cistola & Small, 1991), their acyl Coenzyme A esters (Richards *et al.*, 1990) and mono-acyl phospholipids (Robinson *et al.*, 1989). It binds to polyunsaturated fatty acids (Anel *et al.*, 1989) and influences the stability (Haeggstrom *et al.*, 1983), biosynthesis (Heinsohn *et al.*, 1987) and conversion of prostaglandins (Dieter *et al.*, 1990). It is involved in the transport of thyroid hormones (Mendel *et al.*, 1990) and tryptophan (Herve *et al.*, 1982) through reversible binding. It modulates the calcium ion stimulation of gluconeogenesis. It is also involved in the transport of pyridoxal phosphate (Fonda *et al.*, 1991), cysteine and glutathione (Joshi *et al.*, 1987) by forming a covalent linkage with these ligands. It hydrolyses substituted aspirins (Kurono *et al.*, 1984), nitrophenyl esters (Kurono *et al.*, 1979) and guanidinobenzoates (Ohta *et al.*, 1988). The tight binding of bilirubin to albumin (Knudsen *et al.*, 1986) prevents the passage of this neurotoxic pigment into the central nervous system in newborns (Esbjorner, 1991) and helps in its delivery to the liver for conjugation and excretion . Albumin is responsible

for the transport and storehousing of many therapeutic drugs in the blood stream (Lindup, 1987). Albumin microspheres are useful carriers of therapeutic agents (Morimoto & Fujimoto, 1985). Serum albumin is also used in peritoneal dialysis, in combating the harmful effect of antibiotics and as scavenger of toxic substances and free radicals (Holt *et al.*, 1984). Albumin is an important constituent of tissue culture media (Barnes & Sato, 1980). It serves as a medium to support growth of microorganisms e.g. bacteria, fungi and yeasts (Callister *et al.*, 1990; Morrill *et al.*, 1990). It has been used as replacement of blood and plasma for the growth of malarial parasites (Nayar *et al.*, 1991). Albumin acts as a carrier for chemotactic factors and affects phagocytosis by leucocytes and macrophages (Echarti & Maurer, 1989). It interacts with cell membrane receptors and thus enhances the intercellular adhesion of the neuroretinal cells. Albumin is also a carrier for haptens. DNP-HSA is often used to test the responsiveness of allergenic systems, such as release of histamine from mast cells (Levi-Schaffer *et al.*, 1990). It has also been used in preservation of tissues such as human kidney and rat liver (Hatzinger & Stevens, 1989; Hellmann *et al.*, 1990). Existence of the condition of 'analbuminemia' though rare but has been observed in many individuals. Clinical manifestation of 'analbuminemia' includes chronic fatigue, hyperlipidemia and in rats an increased susceptibility to cancer (Kakizoe & Sugimura, 1988).

Binding of ligands to albumin

Albumin binds to a wide variety of ligands both exogenous as well as endogenous in

nature which include metal ions, drugs, fatty acids, bilirubin, anesthetics and peptide hormones etc. (Carter & Ho, 1994). Figure 2 illustrate the binding site of various ligands on HSA as proposed by Carter and Ho (1994). Most ligands are bound reversibly with an association constant ranging between 10^4 to 10^8 M^{-1} (Carter & Ho, 1994). Binding sites have been primarily studied by equilibrium dialysis or spectroscopic method and the data are generally analyzed by Scatchard method (Scatchard, 1949). Some of the important informations regarding these binding sites have been gained from the recently determined crystal structure of HSA (He & Carter, 1992; Sugio *et al.*, 1999). The two strong binding sites reside in subdomains IIA and IIIA corresponding to Site I and Site II respectively as proposed by Sudlow and his group (Sudlow *et al.*, 1976). Although vast majority of ligands bind to both sites but subdomain IIIA (Site II) seems to possess the primary binding activity for albumin whereas subdomain IIA (Site I) is more specialized (Carter & Ho, 1994). The charge distribution is quite similar for both IIA and IIIA binding pockets but these sites still impart specialized selectivity. In both the subdomains, there is an asymmetric charge distribution leading to a hydrophobic surface on one side and basic or positively charged surface on the other (Carter & Ho, 1994). Site II (subdomain IIIA) shows preference for small aromatic carboxylic acids whereas Site I (subdomain IIA) is primarily the locus for heterocyclic compounds having negative charge. This is evident from the observations suggesting an unusually high reactivity of Lys-199 which can be acetylated by aspirin (Walker, 1976) and considered to be the major site of conjugation to benzopenicillin groups (Yvon & Wal, 1988) and also appears to be the site for non-

enzymatic glycosylation (Day *et al.*, 1979). Additionally, Lys-240 and Lys-238 (Site I in subdomain IIA) of HSA and BSA respectively have been shown to be important in forming a salt bridge with carboxyl group of bilirubin (Jacobsen, 1978) and these lysine residues are supposed to be buried in the protein interior (Tayyab & Qasim, 1987; Mir *et al.*, 1992). Much of the chemistry of ligand binding to albumin has been gained from the observations on the binding of 2,3,5-triiodobenzoic acid which has a moderate and equal affinity for subdomains IIA and IIIA but with distinct geometry (Carter & Ho, 1994). The absence of a similar binding cavity in subdomain IA is interesting which has been explained from the crystal structure of HSA. A non-helical section of polypeptide allows helix -3 to markedly increase its packing angle with helix-4 and thereby effectively eliminating any binding pocket with subdomain IA (Carter & Ho, 1994).

Metals primarily bind to the N-terminal region of albumin (Figure 2) which has high affinity for Cu (II), Ni (II), Hg (II), Au (I) and Ag (II). Albumin has weaker affinity for calcium and zinc (Carter & Ho, 1994). Histidine at position 3 is involved in high affinity binding of Cu (II) and Ni (II). Cys-34 also binds to a number of metals including Au, Ag, Hg, Cd and to a lesser extent Cu. The Cys-34 is conserved in mammalian serum albumins.

Albumin binds a variety of saturated and unsaturated fatty acids and the number of bound fatty acids depends on the chain length. An average of six fatty acids can bind to one albumin molecule (Carter & Ho, 1994) but under normal conditions, albumin carries

one or two fatty acids (Peters, 1985). Hamilton *et al.* (1991) suggested that one primary fatty acid binding site exists in subdomain IB (Figure 3) and two additional binding sites in domain III which is in agreement with the fact that major sites reside in domains I and III of albumin (Peters, 1985), possibly in B subdomains (Brown & Shockley, 1982). Binding of fatty acids leads to significant conformational changes in albumin which produces slight opening of the interface between the two halves of the molecule and a rotation of domain I (Carter & Ho, 1994) leading to more exposure of environment around Cys-34 to the solvent which agrees well with the increased oxidation of Cys-34 as a function of fatty acids' binding (Takabayashi *et al.*, 1983).

An enormous number of synthetic drugs bind to albumin (Figure 2) which caries them to the liver where they are degraded and excreted out. Drug-induced displacement of bound bilirubin from albumin has prompted an intensive research in the area of drug protein interaction because of its relationship to kernicterus in newborn infants (Brodersen, 1974; Karp, 1979; Brodersen & Ebbesen, 1983; Brodersen & Robertson, 1989; Robertson *et al.*,1991). A large number of drugs, if given to infants may displace bilirubin from albumin leading to increased bilirubin concentration in blood whereby the unbound pigment may enter the brain causing bilirubin encephalopathy or kernicterus and other late stage brain damage (Brodersen, 1974; Robertson *et al.*, 1988; Fink *et al.*, 1987). Sulfonamide is one major class of drugs which has been shown to displace firmly bound bilirubin both *in vitro* and *in vivo* (Schutta & Johnson, 1971; Bratlid, 1976) and increases the risk bilirubin encephalopathy / kernicterus. The other class of

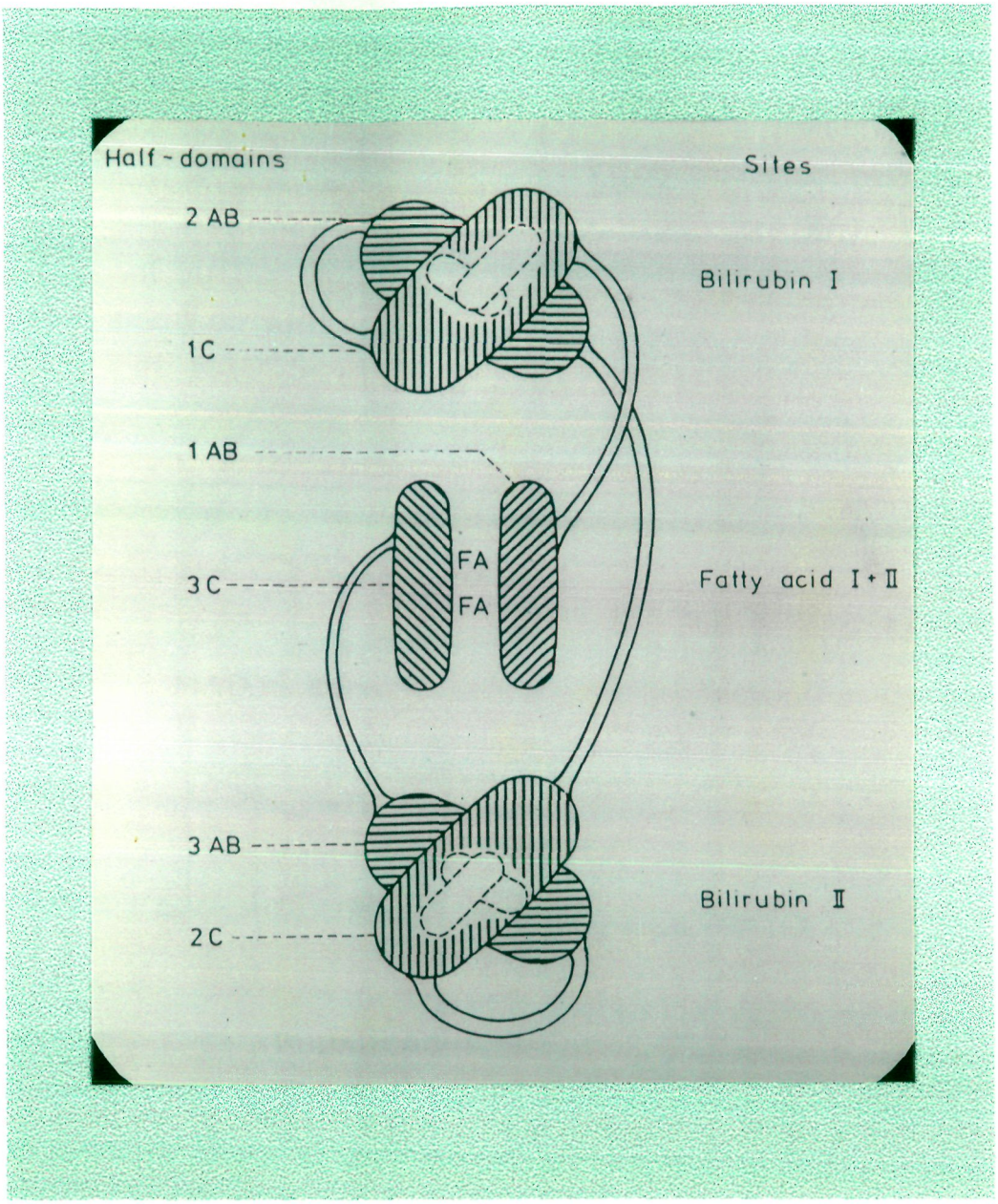


Figure 3. A model of albumin molecule showing six half-domains which are combined to form two sites for binding of bilirubin and one for fatty acids (Taken from Brodersen, 1979). According to the new nomenclature, half-domains AB and C now refer to subdomains A and B respectively (Carter & Ho, 1994).

drugs which displace firmly bound bilirubin from albumin leading to increased risk of bilirubin encephalopathy include, hypnotics and sedatives, drugs used in psychiatric disorder, in Parkinson's disease, narcotics, analgesic-antipyretic and anti-inflammatory, antihistamines, drugs used in chemotherapy of leprosy, tuberculosis and hypoglycemia and various antibiotics etc. (Brodersen, 1979).

Bilirubin encephalopathy / kernicterus

Bilirubin is a red bile pigment produced by the catabolism primarily of hemoglobin in mammals (Schmid & McDonagh, 1975). Approximately 3-4mg bilirubin / kg of body weight is produced per day in healthy adults. In infants, production of bilirubin is high (6-8mg/ kg) because of greater red blood cell mass and shorter red blood cell life span (Gourley, 1997). Increased bilirubin production may lead to the development of hyperbilirubinemia as observed in case of neonatal jaundice (Gourley, 1997). The toxic effects of bilirubin are checked by albumin which binds bilirubin and carries it to the liver for conjugation with glucuronic acid and subsequent excretion in the form of bile from the body and therefore acts as a biologic buffer against bilirubin encephalopathy (Brodersen, 1974; 1979; McDonagh & Lightner, 1985). Excess free or unbound bilirubin in blood is toxic to various cells and tissues including brain (Brodersen, 1979). The condition is more severe in case of human infants due to increase risk of bilirubin entry into the brain because of immature blood brain barrier (Hansen & Bratlid, 1986). Clinical

evidences suggest that there may be two kind of bilirubin effects on brain. When many infants appear to suffer transiently such as lethargy, anorexia and changes in evoked potential, the syndrome is called as bilirubin encephalopathy (Hansen & Bratlid, 1986). However, when the result is death or permanent neurological sequelae, the term kernicterus (yellow staining of basal ganglia) is commonly used (Schmorl, 1904). Increased entry of bilirubin into brain is associated with reduced bilirubin binding capacity of albumin as that of neonatal albumin, hypoalbuminemia, presence of displacing agents and opening of blood brain barrier due to hyperosmolality, hypercarbia and asphyxia. Recently in experimental animals, it has been observed that albumin-bound bilirubin may also enter the brain and thereby further aggravate the risk of bilirubin encephalopathy which may be further facilitated by slight opening of blood brain barrier (Hansen & Bratlid, 1986; Hansen *et al.*, 1987; 1989).

The effects of bilirubin on neurophysiologic processes have only been studied to a limited extent. It has been observed that in the cells, bilirubin is bound to enzyme carrying membranes such as those of mitochondria (Brodersen, 1979). It shows wide spread effects on protein phosphorylation by inhibiting various enzymes, namely, cAMP-, cGMP-, Ca^{++}-calmodulin- and Ca^{++}-phospholipid-dependent protein kinases (Hansen *et al.*, 1996). It also inhibits phosphorylation of histones in the brain of 3-4 days old rabbit (Morphis *et al.*, 1982). Inhibitory effects of bilirubin on *in vivo* incorporation of leucine into protein in adult Gunn rat brain have also been reported (Greenfield &

Majumdar, 1974).

Structure of bilirubin

Bilirubin is a bichromophoric tetrapyrrole (Figure 4A) with several groups capable of forming hydrogen bonds, namely two carboxyl, two pyrrole NH, two lactam NH and two lactam carbonyls (Bonnett *et al.*, 1978). There are also several hydrophobic groups viz. four methyls, two vinyls, two ethylenes and one central (-$^{10}CH_2$-) methylene group. The two halves of the parent molecule (each half consists of two pyrrole groups called as dipyrrole / pyrromethanone unit) which exist in (Z,Z) configuration (Figure 4A) can be rotated at –^{10}C- carbon atom to produce a wide variety of rather different and distinctive conformational structures. Upon exposure to light, bilirubin is transformed into different photoisomers such as *E,Z- or Z,E-* bilirubin due to rotation of either of the end pyrrole ring (Figure 4C) and into *E,Z-* or *E,E*-cyclobilirubins upon prolonged photoirradiation due to *endo-vinyl* cyclization (Brodersen, 1979; McDonagh *et al.*, 1982a; 1982b; 1989; Lamola *et al.*,1983). The photobilirubins are water soluble compounds unlike parent bilirubin molecule which is poorly soluble in aqueous solvents. Insolubility of bilirubin in aqueous solvents can be ascribed to the tendency to form intramolecular hydrogen bonds (Figure 4B) thereby rendering two of its carboxyl groups inaccessible to the solvents (Bonnett *et al.*, 1978). The solubility of bilirubin increases with the increase in pH of the solution or increase in solvent polarity being higher in dimethyl sulfoxide and chloroform (Brodersen, 1979).

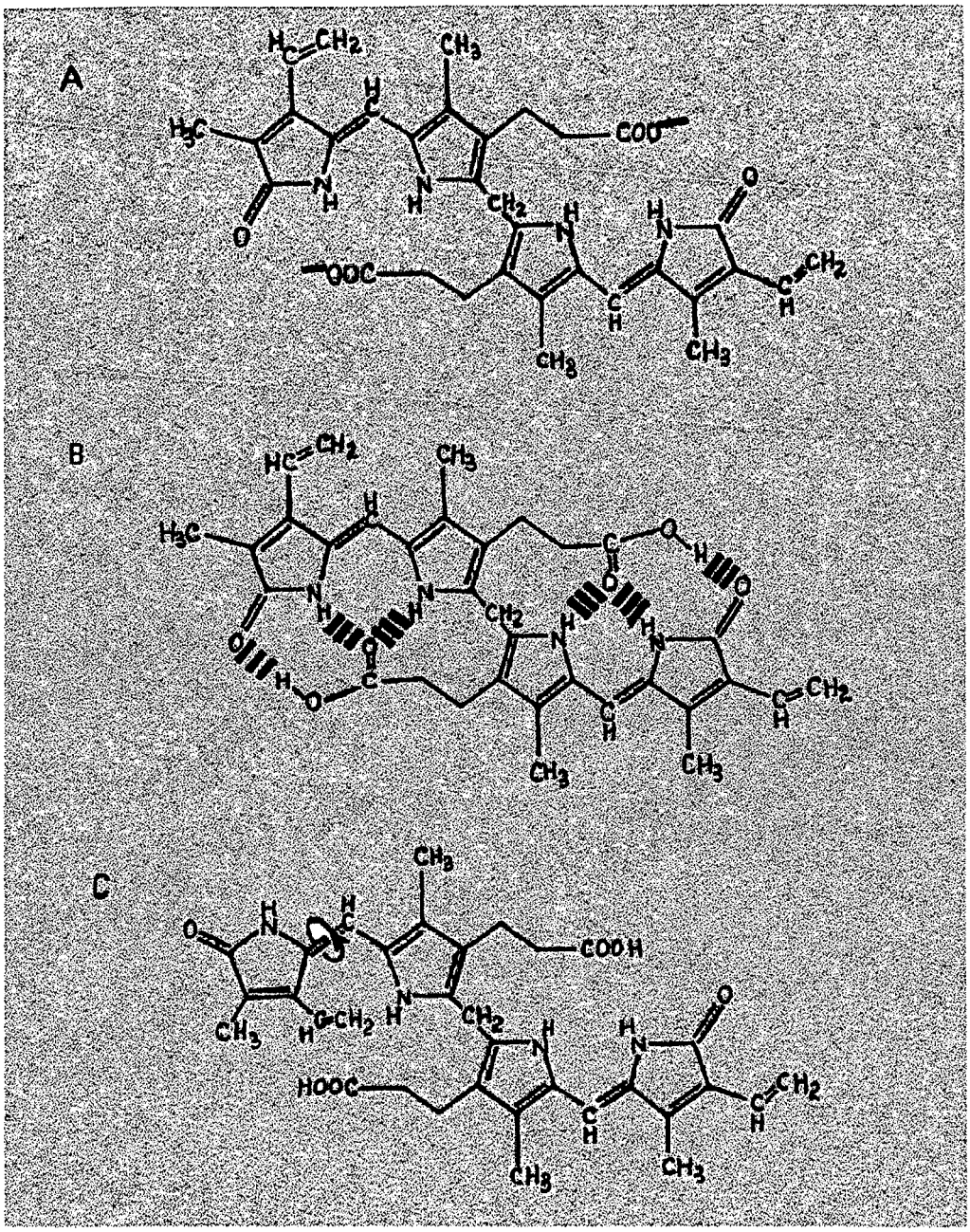


Figure 4. Bilirubin IX-α as (A) dianion and (B) acid, intramolecularly hydrogen bonded, (C) Photoisomerization of parent compound leads to the formation of *E,Z*- or *Z,E*-bilirubins and possibly *E,Z*- or *EE*-cyclobilirubins (Adapted from Brodersen, 1979).

Bilirubin- albumin binding

Albumin has one strong (primary site) and one weak bilirubin binding site (Figure 3) but only primary binding site is of physiological importance because of the safe delivery of the bound pigment to the liver (Peters, 1992). Affinity labeling as well as studies of bilirubin binding to proteolytic fragments and to albumin derivatives suggest that high affinity bilirubin binding site resides near loop 4 of subdomain IIA (Figure 3) encompassing amino acid residues 186-248 (Jacobsen, 1972; 1976; 1978; Reed *et al.*, 1975; Kuenzle *et. al.*, 1976). These results are in agreement with the observations suggesting that in HSA, first bilirubin molecule binds near tryptophan residue at a distance of ~27 Å (Berde *et al.*,1979) as shown in Figure 5. The second relatively weaker binding site resides in domain III as shown in Figure 3. Bilirubin at the primary site binds in a highly specific way in the form of dianion (Figure 4A). It has been suggested that the two dipyrrole chromophores of the bilirubin molecule bind to two different mobile parts (half domains) of albumin (Figure 3) such that the chromophores can be rotated relative to each other upon binding of bilirubin to albumin (Jacobsen & Brodersen, 1983). Kinetic studies have revealed that the binding is rapid followed by several relaxation steps of conformational changes as seen in the light absorption spectra of bilirubin upon binding to albumin (Jacobsen & Brodersen, 1983).

Although the binding of bilirubin to albumin has been studied for nearly 25 years using circular dichroism (CD), nuclear magnetic resonance (NMR) and absorption spectroscopy (Harmatz & Blauer, 1975; Kaplan & Navon, 1981; 1982; 1983; Lightner *et*

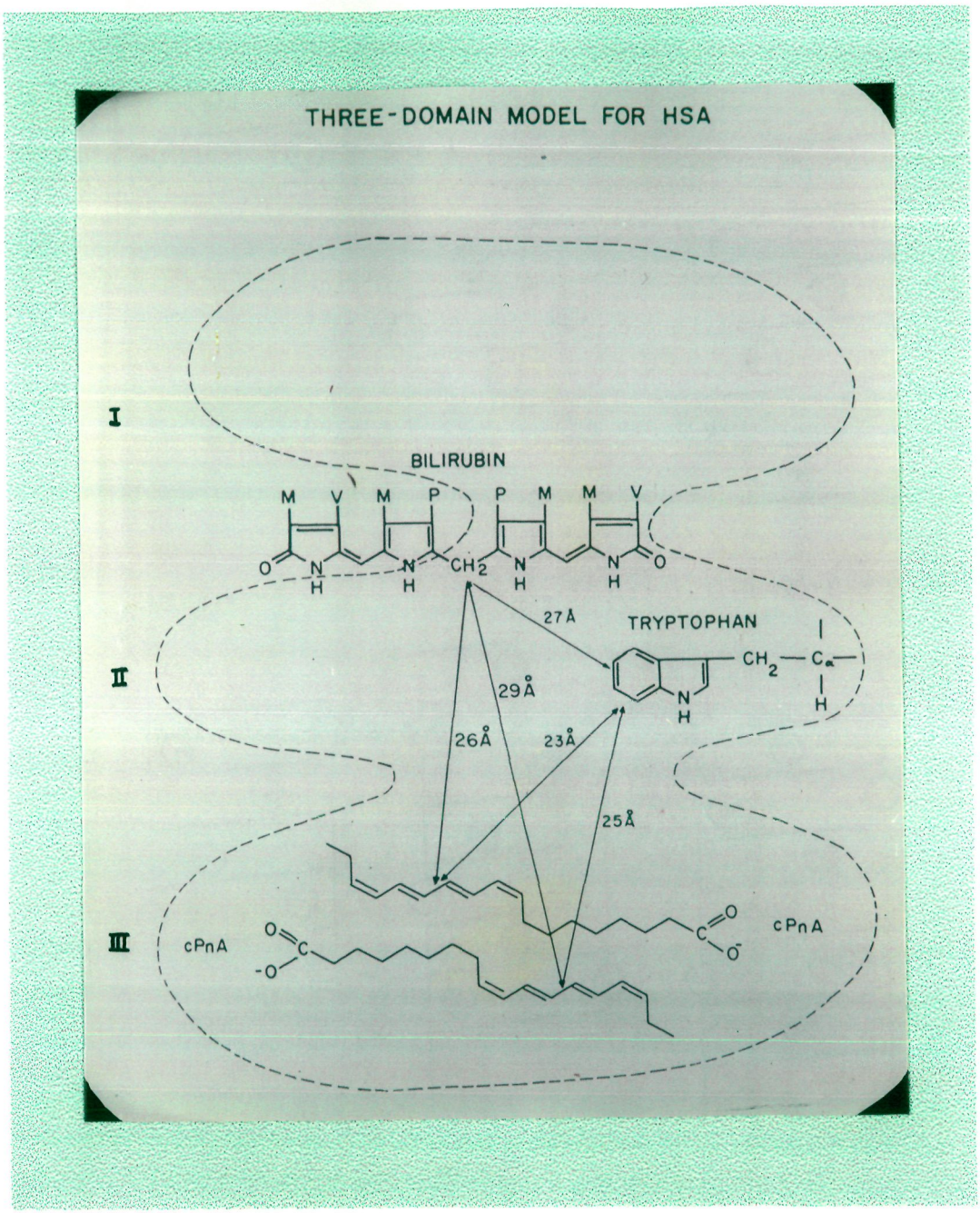


Figure 5. A model of human serum albumin showing the position of bound bilirubin in the crevasse between domains I & II and its average distance from the lone tryptophan-214 (Taken from Berde *et al.*,1979).

al., 1986; 1988), but the conformation of bound bilirubin and nature of binding site on albumin remains poorly understood. It is thought that bilirubin binds to albumin in a folded dissymmetric conformation (Figure 6) akin to that observed in crystalline bilirubin (Nogales & Lightner, 1995). Bilirubin in solution exists in two enantiomeric conformations A and B (Figure 6) which are in dynamic equilibrium $A \rightleftharpoons B$ (Manitto & Monti, 1976; Kaplan & Navon, 1983). This equilibrium process is accompanied by breaking and remaking all of the six possible intramolecular hydrogen bonds. Albumin binds exclusively to one of the enantiomers depending on the species chosen and induces circular dichroism (CD) in the visible region, in the bound bilirubin. The induced CD spectra may be characterized by bisignate CD Cotton effects (CDCEs) or monosignate CDCE usually of longer wavelength and negative sign (Harmatz & Blauer, 1975). In case of bilirubin complexed with HSA, CD spectra is characterized by the bisignate CDCEs having minima (–) at shorter wavelength and maxima (+) at longer wavelength (Harmatz & Blauer, 1975; Blauer *et al.*, 1977; Lightner *et al.*, 1986; 1988). The observed bisignate CDCEs are due to chromophore - chromophore interaction of the two locally excited states of the twin pyrromethanone chromophores i.e. exciton splitting (Harada & Nakanishi, 1983). This exciton splitting is especially dependent on the magnitude and directions of the two electric transition moments / electric dipole moments involved (Hansen & Bouman, 1980; Harada & Nakanishi, 1983) as shown in Figure 6. The sign order in a bisignate CD spectrum depends on the relative helical orientation of the two pyrromethanone electric dipole moments. A right handed helicity (positive (P)

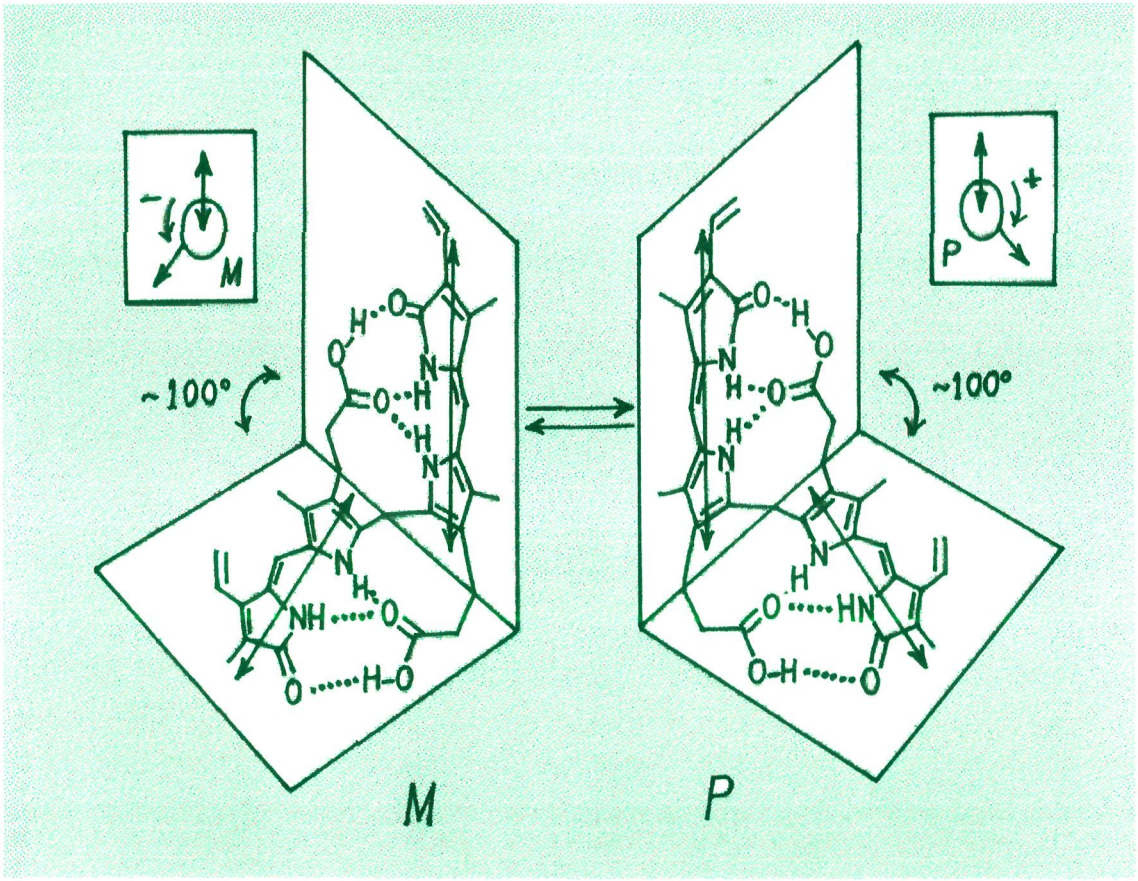


Figure 6. The two ridge-tile conformational enantiomers of bilirubin.The double-headed arrows passing through the dipyrrinone chromophores indicate the orientation of electronic transition dipoles of each dipyrrinone. Insets show the relative helicities (***M***, minus or ***P***, plus) of the dipoles (Boiadjiev & Lightner, 1997).

chirality) of the transition moments generates a (+) longer wavelength Cotton effect followed by a (–) shorter wavelength Cotton effect as observed in case of bilirubin-HSA complex. On the other hand, left handed helical orientation (negative (*M*) chirality) of the electric dipole moments produces inverted CDCEs (Harada & Nakanishi, 1983) as observed in case of bilirubin-BSA complex (Harmatz & Baluer, 1975).

Bilirubin-albumin binding is facilitated by several noncovalent forces such as hydrogen bonds, salt linkages, π–π van der Waals attractions and generalized hydrophobic interactions (Jacobsen, 1977; Brodersen, 1979; 1986). Although, combination and topography of these binding elements dictate the three dimensional shape and chirality of the bound pigment (Lightner *et al.*, 1986; 1988) but salt linkages (-$COO^{-}\cdots{}^{+}H_3N$-) and associated intermolecular hydrogen bonds (Figure 7) appear to be of particular significance in the pigment binding, orientation and enantioselectivity rather than binding alone (Lightner *et al.*, 1986; 1988). In different serum albumins, there exist variations in the combination and topography and to some extent the contribution of these binding elements at the pigment binding site as reflected from the differences in the CD spectra of their complexes with bilirubin (Harmatz & Blauer, 1975; Khan *et al.*, 2000). According to Blauer & Wagniere (1975), the two chromophores of bilirubin in the complex with albumin are fixed at a dihedral angle (Figure 6) formed by rotation around central carbon $-{}^{10}CH_2$- atom (Blauer & Wagniere, 1975; Jacobsen & Brodersen, 1983). Differences in the CD spectra of bilirubin bound to different serum albumins reflect the differences in the dihedral angle between the two pyrromethanone units (Harmatz &

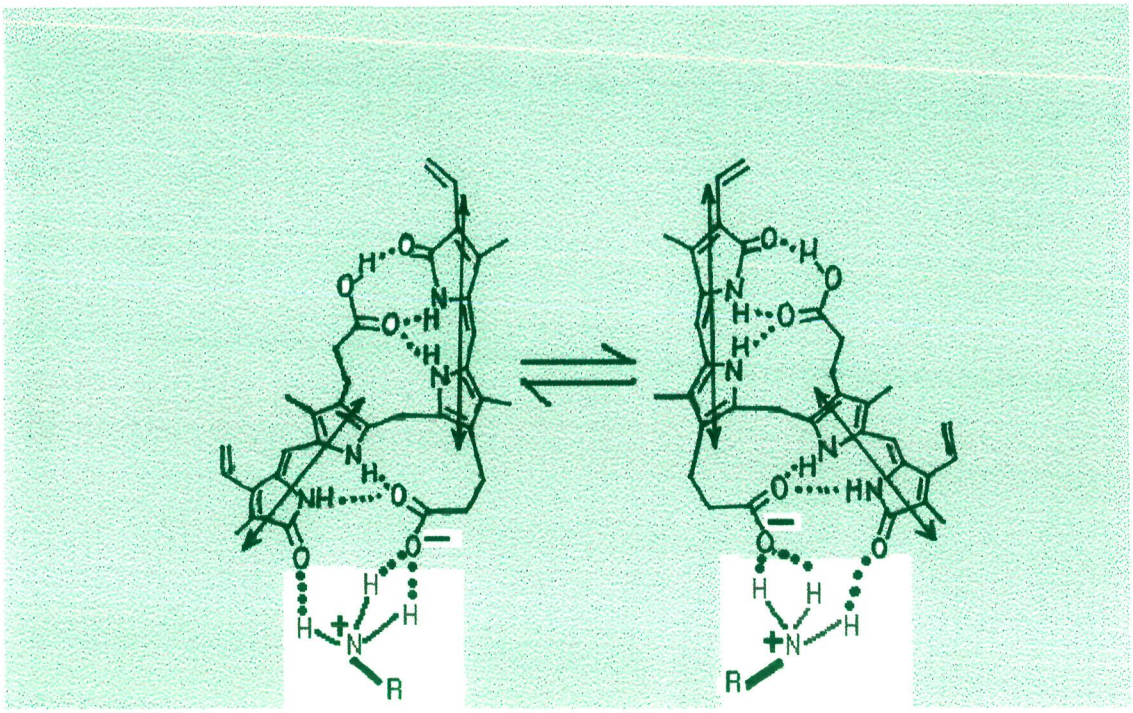


Figure 7. Mono-amine salt complexes of intramolecularly hydrogen bonded enantiomers of bilirubin. Figure also shows associated intermolecular hydrogen bondings from the primary ammonium ion of the amino groups possibly of lysine residues in the case of serum albumin-bilirubin interaction (Taken from Lightner *et al.*, 1988).

Blauer, 1975).

From a brief review of literature presented above, it is clear that although nature has manifested a high degree of sequence homology, similar domain and subdomains organization in different serum albumins but an extensive *in vitro* studies revealed subtle differences in bilirubin binding to these serum albumins with respect to both conformation and affinity of the pigment (Harmatz & Blauer, 1975; Robertson *et al.*, 1990). Moreover, both *in vitro* and *in vivo* studies of phototherapy also showed species dependent differences in the photoproducts formation (McDonagh *et al.*,1982a; 1982b; Onishi *et al.*, 1986). Among various forces involved in bilirubin-albumin interaction, salt linkages existing between carboxyl groups of bilirubin and ε-NH_2 groups of lysine residues of albumin predominate and these lysine residues are believed to be buried in the protein interior having altered p*K* values (Tayyab & Qasim, 1987; Mir *et al.*, 1992). Although, the role of internal lysine residues in bilirubin binding has been studied using BSA, a detailed investigation with respect to the affinity and conformation of bilirubin bound to different serum albumins is lacking. In order to understand the mechanism of bilirubin encephalopathy and to develop various preventive measures, selection of an animal is futile unless the animal albumin binds bilirubin in the similar manner as human albumin binds. In view of this, five different mammalian serum albumins from human (HSA), rabbit (RbSA), goat (GSA), sheep (SSA) and buffalo (BuSA) were selected and their conformational and bilirubin binding properties including conformational stability of albumin-bound bilirubin were studied after modifying the internal lysine residues with acetic anhydride, succinic anhydride and O-methylisourea.

Materials & Methods

MATERIALS AND METHODS

[A] MATERIALS

1. Proteins

Various marker proteins viz. γ-globulin (lot 72H9312), catalase (lot 120H7060), bovine serum albumin (BSA) (lot 124F0752), ovalbumin (lot 13H7025), ribonuclease (lot 123H1133) and myoglobin (lot 55H7015) were purchased from Sigma Chemical Company, MO, USA. Different serum albumins, namely, human, sheep, goat, rabbit and buffalo serum albumins were isolated from their respective plasma by ammonium sulfate fractionation method (Tayyab & Qasim, 1990) and purified to homogeneity by Seralose-6B gel chromatography.

2. Ligands used in binding studies

Bilirubin-IXα, ultra pure was purchased from Gedeon Richter, Hungary and was used without further purification. Chloroform (99%), analytical grade, was the product of Sisco Research Laboratories, Mumbai, India.

3. Reagents used in protein modification

Citraconic anhydride (lot 910425092) and tri-nitrobenzene sulfonic acid (lot 28999) were the products of Pierce Chemical Company, IL, USA. O-Methylisourea (lot

85H0142) and guanidine hydrochloride (lot 94H5702) were obtained from Sigma Chemical Company, MO, USA. Chemically pure samples of succinic anhydride and acetic anhydride were supplied by Sisco Research Laboratories, Mumbai, India. Hydroxylamine hydrochloride was obtained from Qualigens Fine Chemicals, Mumbai, India.

4. Reagents used in gel chromatography

Seralose-6B (Batch No. B716063) was purchased from Sisco Research Laboratories, Mumbai, India and blue dextran 2000 (type D-5751; lot 62H0796) was supplied by Sigma Chemical Company, MO, USA.

5. Reagents used in gel electrophoresis

Acrylamide (3x crystallized) and N, N'-methylenebisacrylamide (3x crystallized) were purchased from Sisco Research Laboratories, Mumbai, India. Trizma base (tris [hydroxymethyl] aminomethane) (lot 14H5717), glycine (lot 113H12551) and coomassie brilliant blue R 250 (type B-7920) were obtained from Sigma Chemical Company, MO, USA. Analytical grade samples of ammonium persulfate, sucrose, glycerol, acetic acid and methanol were procured from Qualigens Fine Chemicals, Mumbai, India. N, N, N', N'-tetramethylethylenediamine (TEMED) was the product

of Fluka, AG, Switzerland. 2-Mercaptoethanol was purchased from E. Merck, Darmstadt, Germany. Bromophenol blue was the product of B. D. H., Poole, England.

6. Reagents used in protein estimation

Analytical grade samples of sodium carbonate, sodium potassium tartarate, copper sulfate, orthophosphoric acid, lithium sulfate, sodium molybdate, sodium tungstate and hydrochloric acid were obtained from Qualigens Fine Chemicals, Mumbai, India. Liquid bromine was procured from Sigma Chemical Company, MO, USA. Folin-phenol reagent was prepared according to the standard procedure (Folin & Ciocalteu, 1927). Copper reagent was prepared fresh by mixing 1 volume of 4% (w/v) sodium potassium tartarate to 100 volumes of 4% (w/v) sodium carbonate and finally adding 1 volume of 2% (w/v) copper sulfate.

7. Miscellaneous

Sodium dihydrogen phosphate, disodium hydrogen phosphate, sodium hydroxide, potassium dichromate, potassium hydrogen phthalate, sodium tetraborate, potassium permanganate, sodium azide and ethanol were of analytical grade and obtained from Qualigens Fine Chemicals, Mumbai, India. Dialysis tubing of 1 inch width was obtained from Sigma Chemical Company, MO, USA. Millipore filters (pore size 0.45

μm) were purchased from Millipore (India) Pvt. Ltd., Bangalore, India. Whatman filter papers (No. 1) were the product of Whatman International Ltd, Maidstone, England. Parafilm 'M' was obtained from American Can Company, CT, USA. pH indicator papers were supplied by Qualigens Fine Chemicals, Mumbai, India. Solid glass beads (5 mm diameter) and glass wool were obtained from S.D. Fine Chemicals, Boisar, India.

All glass distilled water was used throughout these studies. All the experiments were performed at room temperature (~ 25 ^{0}C) unless otherwise stated.

[B] METHODS

1. pH measurements

pH measurements were carried out on an Elico digital pH meter, model LI 610 using a combined electrode, type CL-51. The least count of the pH meter was 0.01 pH unit. The pH meter was routinely calibrated at room temperature with either 0.05 M potassium hydrogen phthalate buffer, pH 4.0 in the acidic range or 0.01 M sodium tetraborate buffer, pH 9.2 in the alkaline range.

2. Determination of protein concentration

Protein concentration was determined either spectrophotometrically or by the method of Lowry *et al.* (1951) using BSA as the standard.

Spectrophotometric method – Protein concentration was determined following absorbance measurement at 280 nm on a Cecil double beam spectrophotometer, model CE 594 and using the value of specific extinction coefficient ($E^{1\%}_{1cm}$) as 5.30 and 6.67 for HSA and BSA respectively (Peters, 1985).

Method of Lowry et al. (1951) – Increasing volumes of stock protein solution (300 µg/ml) in the range of 0.1-1.0 ml were taken in different test tubes and the volume in

each tube was made to 1.0 ml, if required, with 0.06 M sodium phosphate buffer, pH 7.0. Then, 5.0 ml of freshly prepared copper reagent was added to all the tubes and the contents were mixed well. After 10 minutes of incubation at room temperature, 1.0 ml of diluted Folin-phenol reagent was added and vortexed. The tubes were incubated for 30 minutes at room temperature and the color intensity was read at 700 nm against a suitable blank prepared in the same way as that of test solution except that instead of protein solution, 1.0 ml of buffer was taken. A calibration curve, thus obtained, between absorbance at 700 nm and amount of protein yielded the following straight line equation (Figure 8):

$$(\text{Absorbance})_{700\ nm} = 1.9 \times 10^{-3}\ (\text{amount of protein, } \mu g) + 0.04 \qquad (1)$$

3. Polyacrylamide gel electrophoresis (PAGE)

Electrophoresis of various serum albumins and their modified derivatives was performed in 8% (w/v) polyacrylamide gel using 0.02 M tris- glycine buffer, pH 8.2, ionic strength 0.02 following the method of Laemmli (1970) under non-denaturing conditions. About 5-8μg of the protein was loaded in 10-20 μl sample buffer and a current of 3-4 mA per well was applied for nearly 2 hours. The gel was stained with the staining solution containing 0.25% (w/v) coomassie brilliant blue in 40% (v/v)

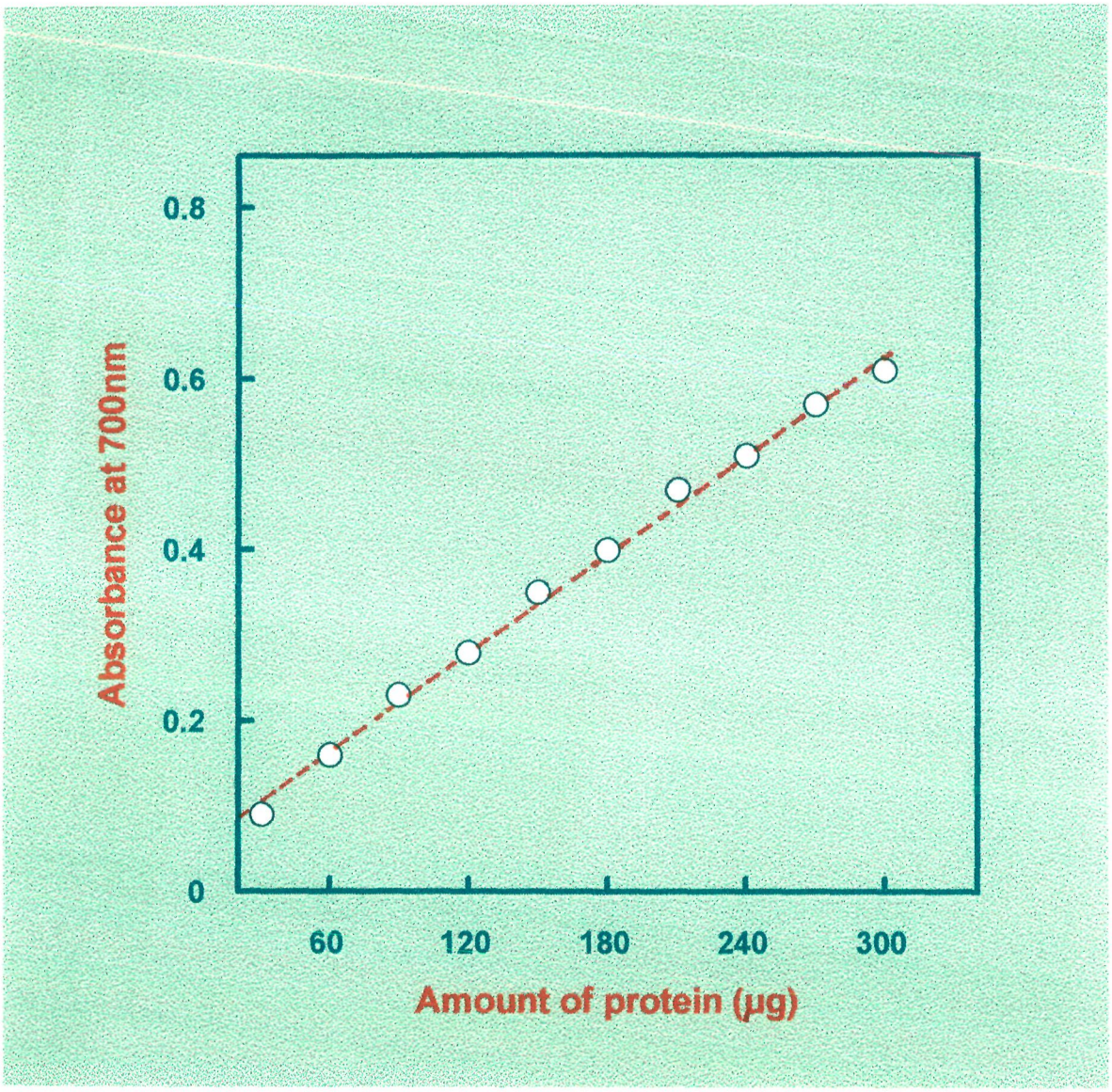


Figure 8. Standard curve for the determination of protein concentration by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard. Straight line was drawn by the method of least squares.

methanol and 10% (v/v) acetic acid and destained with 10% (v/v) acetic acid solution containing 10% (v/v) methanol at 37^0C.

4. Covalent blocking of internal lysine residues of different serum albumins

Acetylation, succinylation and guanidination of internal lysine residues of different serum albumins were carried out according to standard procedures (Riordan & Vallee, 1967; Klotz, 1967; Kimmel, 1967) using a double modification approach (Mir *et al.*, 1992). The surface lysine residues (completely as well as partially exposed) of these albumins were first protected by using a reversible modifier, citraconic anhydride (Dixon & Perham, 1968). For each albumin, the desired concentration of citraconic anhydride to covalently block ~80% surface lysine residues was obtained by treating each albumin separately with increasing anhydride concentrations. The reaction was performed in 0.1 M sodium phosphate buffer, pH 8.0 for about 30-40 minutes. The pH of the reaction mixture was maintained at pH 8.0 by simultaneous addition of 0.5 N NaOH. After completion of the reaction, the preparation was dialysed against 0.06 M sodium phosphate buffer, pH 8.0 to remove excess modifier. The extent of citraconylation was determined by TNBS reaction method (Hebeeb, 1966) and percentage modification was calculated using the following straight line equation:

$$\% \text{ modification} = (1 - m / m_o) \times 100 \qquad (2)$$

where m and m_o are the slopes of the plots between absorbance at 340 nm and amount

of protein, obtained with native and modified albumins drawn by the method of least squares. Using appropriate concentration of citraconic anhydride, about 80% lysine residues of each albumin were modified in the same way as described above. These 80% citraconylated albumin preparations were unfolded with 6M guanidine hydrochloride to expose internal unmodified lysine residues and divided into four parts. The three of the four parts of each citraconylated albumin were then treated with acetic anhydride, succinic anhydride and O-methylisourea respectively to modify internal (~20%) lysine residues. The fourth part was kept for testing the reversibility and consequences of citraconylation on protein conformation, if any. Acetylation, succinylation and guanidination were carried out as described earlier (Riordan & Vallee, 1967; Klotz, 1967; Kimmel, 1967). These preparations were then thoroughly dialysed against 0.05 M sodium acetate buffer, pH 3.5 at 10°C for 96 hours to remove citraconyl groups. The extent of acetylation, succinylation or guanidination was determined by TNBS reaction method (Habeeb, 1966). The homogeneity of these preparations was checked by PAGE (Laemmli, 1970) as well as Seralose-6B gel chromatography (Tayyab *et al.*, 1991). For the quantification of modification, total number of amino groups was taken as 60 in both HSA and RbSA and 59 in SSA (Peters, 1985; Carter & Ho, 1994; Peters, 1996). Based on the similarities in the molecular properties of GSA and BuSA with those of HSA, the total number of amino groups in these albumins was also assumed to be 60. The site specificity of different modifiers used in this study, towards amino groups has been well documented

(Riordan & Vallee, 1967; Klotz, 1967; Kimmel, 1967). However, modification of other residues, if any, was made reversible in all these preparations following standard procedures (Gounaris & Ottesen 1965; Riordan & Vallee, 1967b; Freedman *et al.*, 1968). Therefore, these preparations were assumed to have only internal lysine residues modified.

5. *Gel chromatography*

Analytical gel chromatography was performed in the same way as described earlier (Tayyab *et al.*, 1991) using a Seralose-6B column (80 x 1.15cm) equilibrated with 0.06M sodium phosphate buffer, pH 7.4. The column was calibrated by passing, at least three times each of the marker proteins, namely, γ-globulin, catalase, BSA, myoglobin and ribonuclease. The mean values of their elution volumes were normalized in terms of distribution coefficient, K_d and available distribution coefficient, K_{av} according to standard procedures (Andrews, 1970). The data were analysed as described by Laurent & Killander (1964) and Ackers (1967) which fit the following straight line equations (4) and (5) respectively (Figure 9):

$$(-\log K_{av})^{½} = 0.077 \text{ (Stokes radius, nm)} + 0.15 \quad (3)$$

$$\text{Stokes radius, nm} = 9.74 \text{ erfc}^{-1}K_d + 0.78 \quad (4)$$

where $\text{erfc}^{-1} K_d$ is the inverse error function complement of K_d.

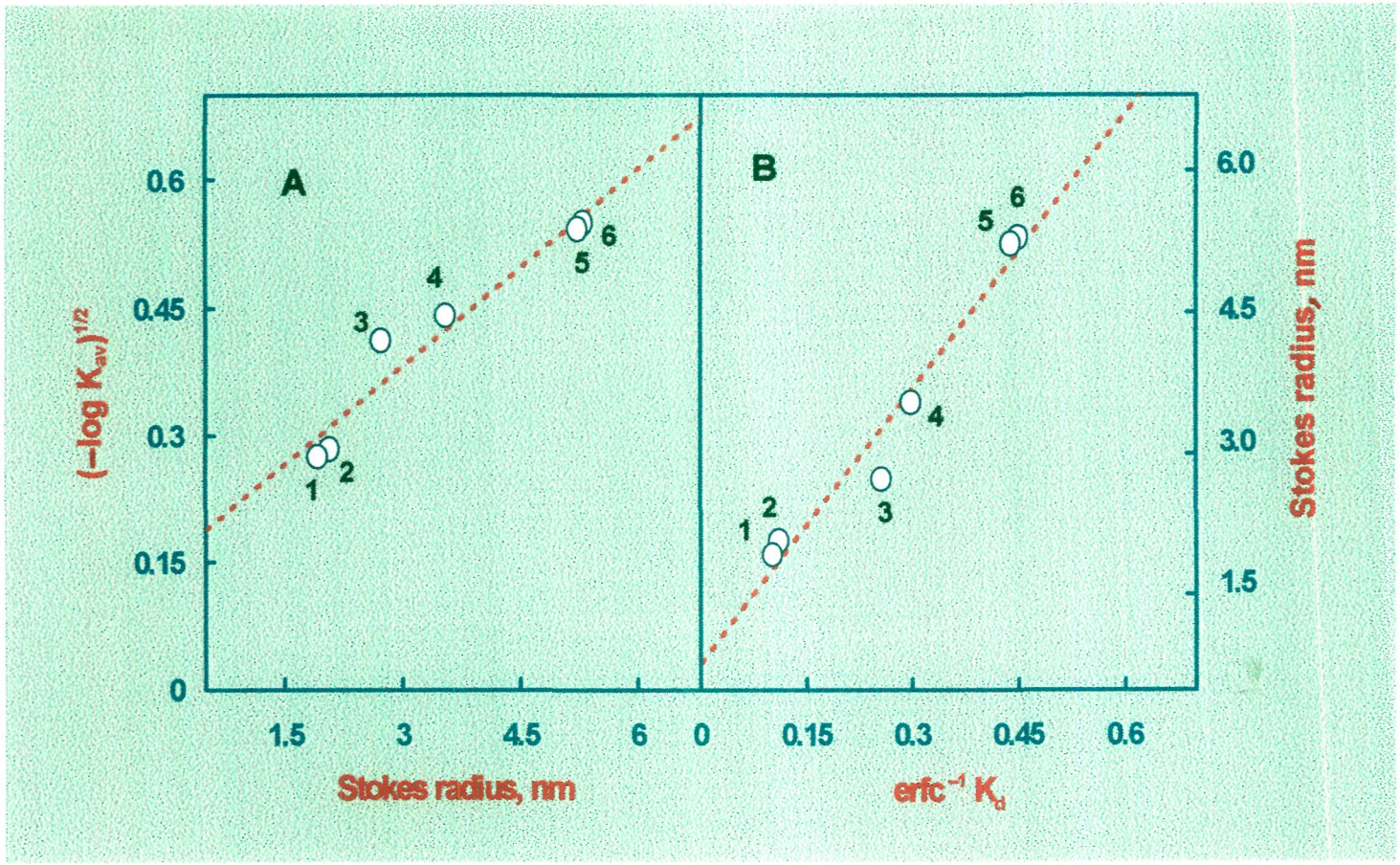


Figure 9. Treatment of gel filtration data of marker proteins according to **(A)** Laurent & Killander (1964) and **(B)** Ackers (1967) for the determination of Stokes radii of the modified derivatives of different serum albumins. The standard proteins used were: (1) ribonuclease (2) myoglobin (3) ovalbumin (4) bovine serum albumin (5) catalase and (6) γ-globulin. Straight lines were drawn by the method of least squares.

6. Circular dichroism spectroscopy

CD measurements were made on a Jasco spectropolarimeter, model J-720 equipped with a microcomputer. The instrument was calibrated with d-10-camphorsulfonic acid. All the measurements were carried out at 25°C with the help of a thermostatically controlled cell holder attached to a Neslab's RTE-110 circulating water bath with an accuracy of ±0.1° C. Far-UV (200-250 nm) CD spectra were taken at a protein concentration of 2 μM with a 1 mm path length cell. Spectra were collected at a scan speed of 20 nm / minute and with a response time of 1 second. Each spectrum was the average of 4 scans. In the near UV region (250-300 nm), CD spectra were measured at a protein concentration of 20 μM with a 10 mm path length cell. The results are expressed as mean residue ellipticity (MRE) in $deg.cm^2.dmol^{-1}$ which is defined as:

$$MRE = \theta_{obs}\,(mdeg)/10 \times n \times l \times C_p \qquad (5)$$

where θ_{obs} is the CD in millidegree; n is the number of amino acid residues; l is the path length of the cell and C_p is the mole fraction. The helical contents of different albumins and their derivatives were calculated from the MRE value at 222 nm using the following equation as described by Chen *et al.* (1972):

$$\%\ helix = (MRE_{222} - 2340 / 30300) \times 100 \qquad (6)$$

The visible range CD spectra were measured in the wavelength range of 350-550 nm at

25 ± 0.1° C using a cell holder of 10 mm path length. The spectra were collected at a scan speed of 500 nm / minute with a response time of 1 second. Each spectrum was the average of 3 scans.

7. Fluorescence spectroscopy

Fluorescence measurements were carried out on a Shimadzu spectrofluorometer, model RF-540 equipped with a data recorder, DR-3 at 25 ± 0.1° C. The fluorescence was recorded in the wavelength range 300-400 nm after exciting the protein solution either at 295 nm exclusively for tryptophan fluorescence or at 280 nm for total protein fluorescence. Induced fluorescence of albumin-bound bilirubin was recorded in the wavelength range 480-580 nm after exciting the samples at the maximum wavelength of their respective absorption spectra (Chen, 1974). The slits were set at 3 and 5 nm for excitation and emission respectively. The path length of the sample cuvette was 1 cm.

8. Bilirubin binding studies

Binding of bilirubin to native and modified albumins was studied using fluorescence, CD and absorption spectroscopy. Bilirubin binding experiments were performed in 0.06 M sodium phosphate buffer, pH 8.0, ionic strength 0.15. Bilirubin solution was

prepared by dissolving ~5 mg solid bilirubin crystals in 1 ml of 5 mM NaOH solution containing 1mM EDTA and immediately diluting it with the above buffer. The concentration of bilirubin was determined spectrophotometrically using a molar absorption coefficient of 47,500 M^{-1} cm^{-1} at 440 nm (Jacobsen & Wennberg, 1974). All the spectral measurements were made after incubating bilirubin-albumin solution at least for 30-40 minutes at 25°C. The spectra were recorded in dim / yellow light to prevent undesired photodegradation of bilirubin.

The fluorescence quench titration of native or modified albumin derivatives with increasing bilirubin / protein molar ratios was performed in a discontinuous manner. To 1 ml of stock protein solution (1-3.5 μM), increasing volumes of stock bilirubin solution (1.2 mM) were added. The total incubation mixture was 3 ml. The molar ratio of bilirubin / protein was varied between 0.1 and 1.0. The fluorescence quench titration data were analyzed by fitting them in Scatchard plot (Scatchard, 1949) as described by Levine (1977) using the following equation:

$$nK_a - K_aQ = Q/[B] = Q/(R-Q)[\text{albumin}]_T \qquad (7)$$

where n is the binding capacity, K_a is the association constant, Q is the fractional quench, [B] is the free bilirubin concentration, $[\text{albumin}]_T$ is the total albumin concentration and R is the bilirubin / albumin molar ratio. The value of K_a was obtained from the slope of the plot between $Q/[B]$ Vs Q.

The visible range CD spectra of bilirubin-albumin solutions were made at a fixed bilirubin (10 μM) / albumin (20 μM) molar ratio of 0.5:1.0. The results are expressed as molar ellipticities $[\theta]_\lambda$ in deg. cm^2. $dmol^{-1}$ which is defined as:

$$[\theta]_\lambda = \frac{10\,\theta}{c.d.} \qquad (8)$$

where θ is the observed ellipticity in degrees; c is the concentration in moles / liter of total bilirubin and d is the path length in decimeters.

Bilirubin binding to albumin was also studied by absorption spectroscopy performed either on a CECIL double beam spectrophotometer, model CE 594 or on a Backman DU-600 spectrophotometer. The absorption spectra of free bilirubin (10 μM) as well as bound to albumin (20 μM) was recorded in the wavelength range 370-550 nm using a cell holder of 1cm path length. The results are expressed in terms of molar absorption coefficient (ε) in $M^{-1}.cm^{-1}$ with respect to the total bilirubin present in solution.

9. Competition between native and modified albumins for bilirubin binding

The switching of bound bilirubin from modified albumin derivatives to their native forms was studied by CD spectroscopy. In brief the protocol is as follows: first bilirubin was incubated with modified derivatives (acetylated as well as succinylated) at a bilirubin / albumin molar ratio of 0.5:1.0 for 30-40 minutes at room temperature in

dark. Then the solution was divided in two parts; in one part required amount of native form of the same acetylated or succinylated albumin species was added to get a bilirubin / albumin molar ratio of 0.5:1 with respect to native albumin and in the second portion, equal volume of reaction buffer was added and both the solutions were then incubated for 30-40 minutes at 25°C in dark. The CD measurements were carried out as described above. To verify the results of competition experiments, same protocol was repeated in reverse order i.e. bilirubin was first incubated with native albumin followed by the addition of modified derivatives (acetylated or succinylated) of the same albumin.

10. Effect of chloroform on the CD spectra of various bilirubin-albumin complexes

The CD spectra of various bilirubin-albumin complexes equilibrated with chloroform were measured in the same way as described above but either prior to or after the addition of bilirubin (10 μM) to albumin (20 μM), protein solution was equilibrated with ~20 mM chloroform for 1 hour under atmospheric conditions (Pu *et al.*, 1993) and the measurements were made after 30-40 minutes incubation of bilirubin-albumin solution at 25°C in dark.

11. Photochemical experiments

For the measurements of photoinduced changes in the fluorescence of bilirubin-HSA complex, samples were continuously irradiated in the spectrofluorometer using a

Xenon lamp of 150 W and a 10 nm bandwidth interference filter centered either at 440 or 468 nm and the fluorescence of the bound bilirubin was simultaneously monitored at 530 nm. The light intensity was supposed to be in the range of 40-50 $\mu W\ cm^{-2}$ (Lamola *et al.*, 1981). The photoinduced alterations in the CD spectrum of bilirubin-HSA complex were measured after irradiating the sample under white fluorescent light generated from a 40 W (FTL) white fluorescent tube. The samples were irradiated for varying time periods i.e. 0, 2, 4, 6, 10, 15 and 30 minutes and then successively transferred to the dark and the CD spectra were recorded as described above.

In the present investigation, the effect of white fluorescent light on the CD spectrum of bilirubin-HSA complex was found to be most effective as compared to the light of a particular wavelength (440 or 468 nm). Therefore, most of the photoinduced alterations in the CD spectrum were measured after irradiating the samples under white fluorescent light as described above.

Results & Discussion

RESULTS AND DISCUSSION

Modification of internal lysine residues

Usually charged residues in proteins are present at the surface where they interact with other charged groups and / or become solvated with aqueous phase in protein hydration spheres. Few charged residues are also found in the protein interior where they can form electrostatic interactions with the oppositely charged residues (Burley & Petsko, 1988). In BSA, approximately 20% of the total lysine residues are buried in the protein interior (Jonas & Weber, 1970). Similarly in HSA, some of the lysine residues are not reactive to tri-nitrobenzene sulfonic acid (Goldfarb, 1970). Further, involvement of buried lysine residues has been reported in bilirubin-albumin interaction (Tayyab & Qasim, 1987; Mir *et al.*, 1992). These observations led us to believe that a few (~18-20%) lysine residues in serum albumins are located in an unusual environment in the protein interior. In view of this we checked the reactivity of lysine residues of different serum albumins, namely, human, goat, sheep, buffalo and rabbit serum albumins towards citraconic anhydride. Figures 10-14A show quantification of modification (citraconylation) of lysine residues of different serum albumins using TNBS reaction method (Habeeb, 1966) at different molar ratios of citraconic anhydride to protein. A progressive increase in citraconylation was observed with the increase in citraconic anhydride concentration which sloped off at higher anhydride concentration (see insets of Figures 10-14A). A comparison of these

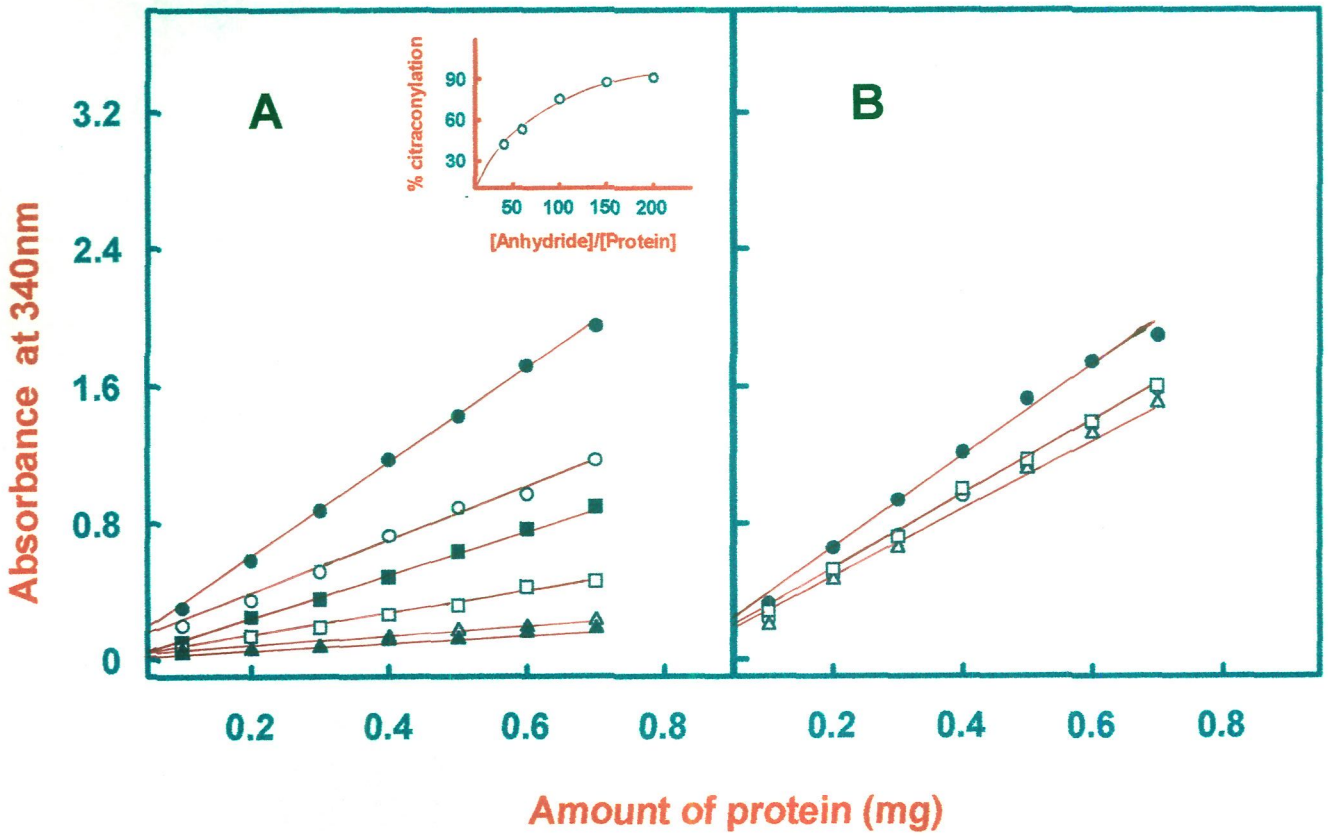


Figure 10. [A] Plots of TNBS color intensity versus protein concentration for HSA (•); HSA treated with increasing molar excess of citraconic anhydride viz. (○) 40, (■) 60, (□) 100, (△) 150 and (▲) 200. Inset shows a correlation between extent of citraconylation and the molar ratio of anhydride to protein. [B] Plots of TNBS color intensity versus protein concentration for HSA (•) and its modified derivatives viz. aHSA (□), gHSA (△) and sHSA (○). Straight lines were drawn by the method of least squares.

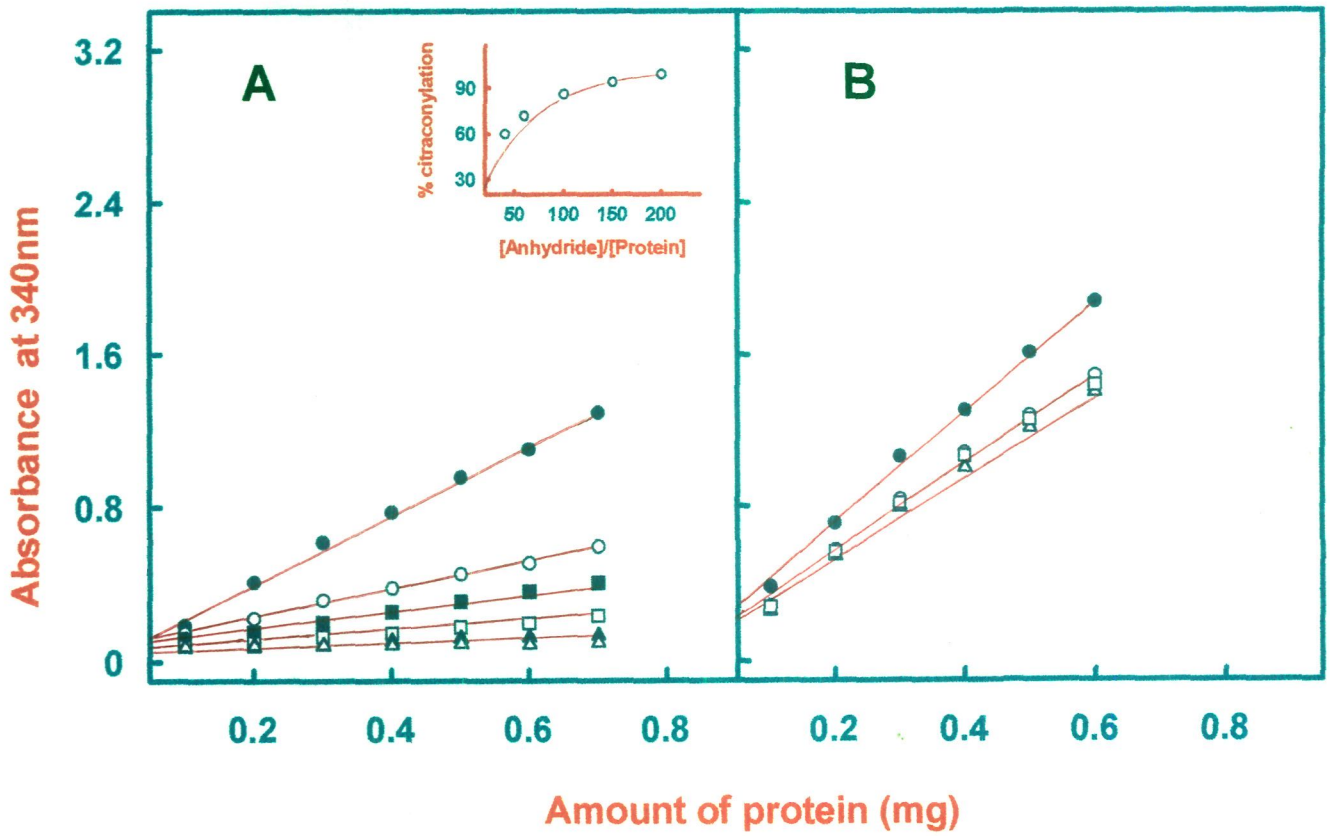


Figure 11. [A] Plots of TNBS color intensity versus protein concentration for RbSA (•); RbSA treated with increasing molar excess of citraconic anhydride viz.(○) 40, (■) 60, (□) 100, (▲) 150 and (△) 200. Inset shows a correlation between extent of citraconylation and the molar ratio of anhydride to protein. [B] Plots of TNBS color intensity versus protein concentration for RbSA (•) and its modified derivatives viz. aRbSA (□), gRbSA (△) and sRbSA (○). Straight lines were drawn by the method of least squares.

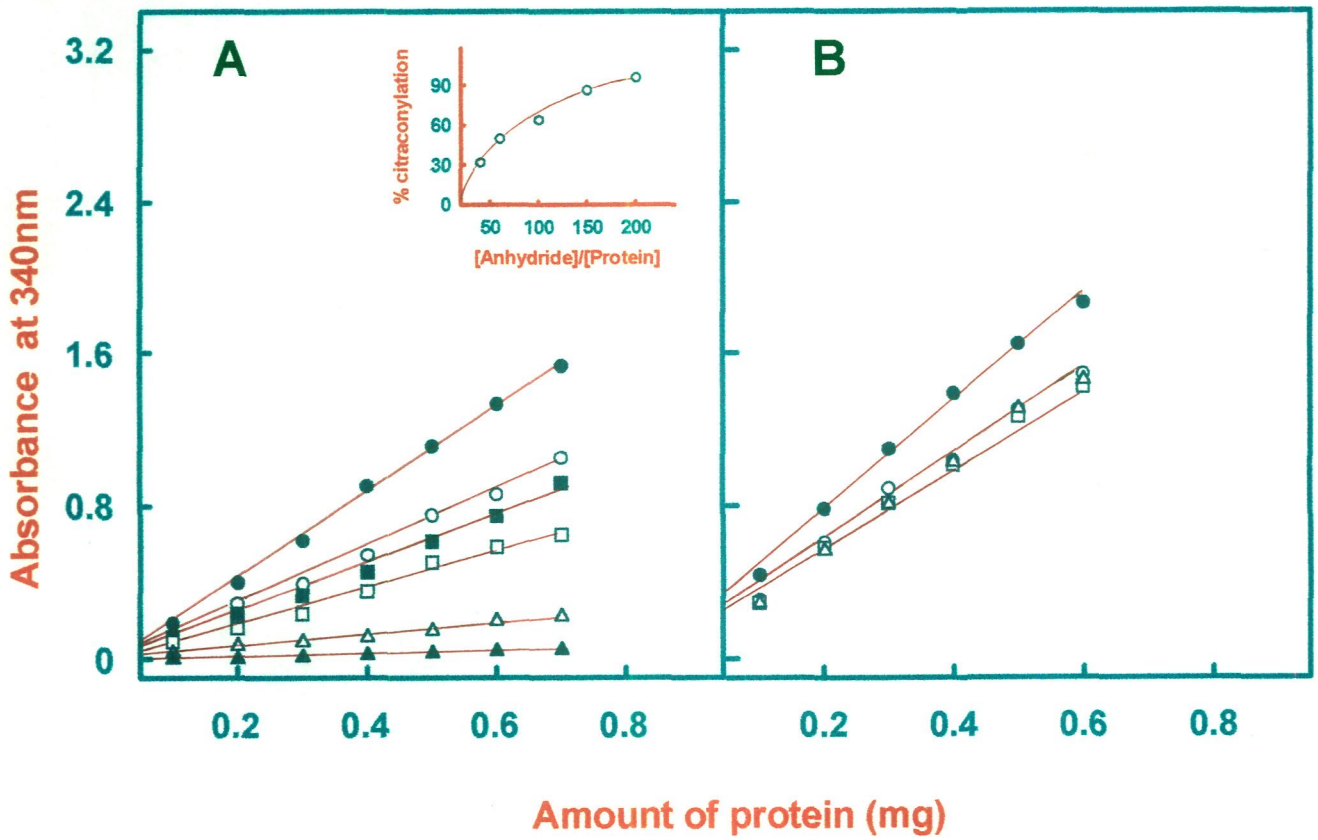


Figure 12. [A] Plots of TNBS color intensity versus protein concentration for GSA (•); GSA treated with increasing molar excess of citraconic anhydride viz. (○) 40, (■) 60, (□) 100, (△) 150 and (▲) 200. Inset shows a correlation between extent of citraconylation and the molar ratio of anhydride to protein. [B] Plots of TNBS color intensity versus protein concentration for GSA (•) and its modified derivatives viz. aHSA (○), gHSA (□) and sHSA (△). Straight lines were drawn by the method of least squares.

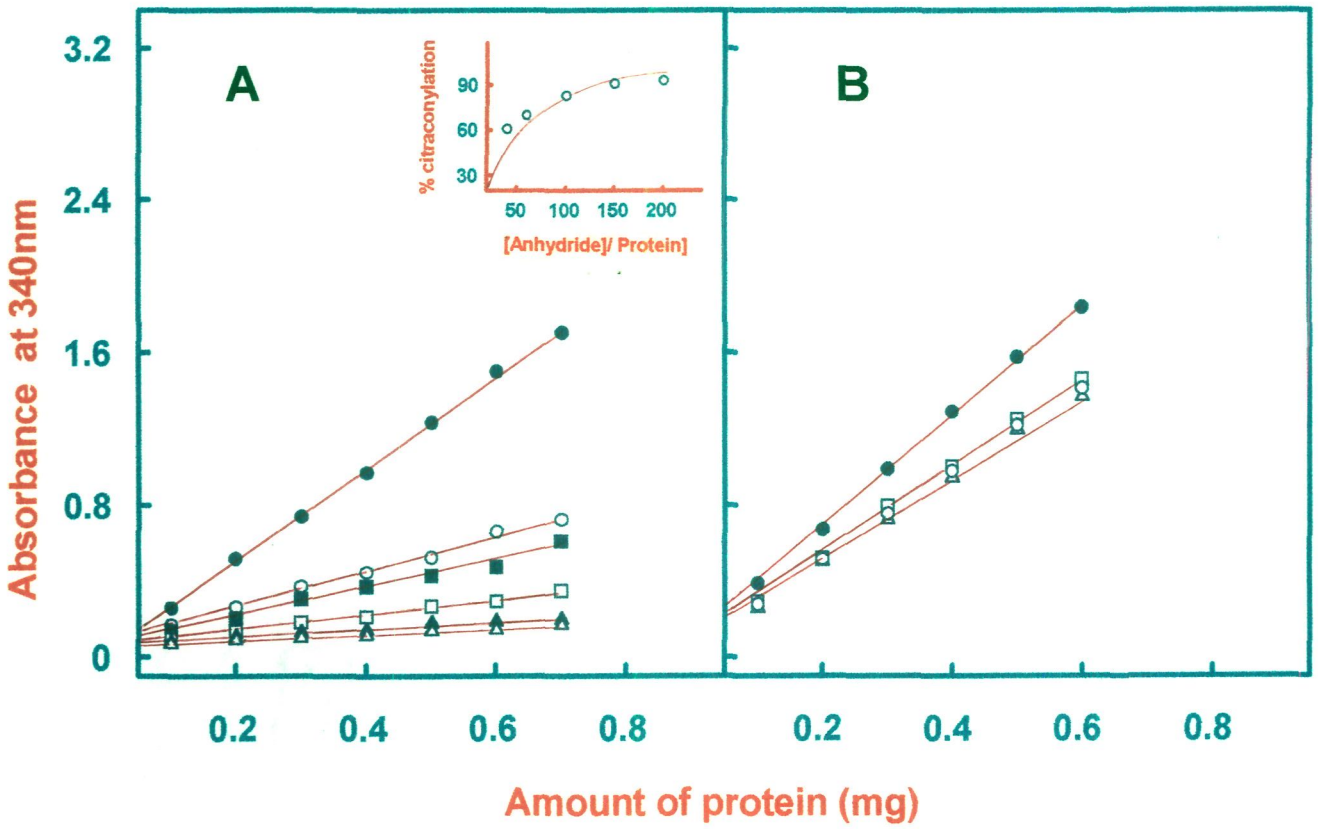


Figure 13. [A] Plots of TNBS color intensity versus protein concentration for SSA (•); SSA treated with increasing molar excess of citraconic anhydride viz. (○) 40, (■) 60, (□) 100, (▲) 150 and (△) 200. Inset shows a correlation between extent of citraconylation and the molar ratio of anhydride to protein. [B] Plots of TNBS color intensity versus protein concentration for SSA (•) and its modified derivatives viz. aSSA (□), gSSA (△) and sSSA (○). Straight lines were drawn by the method of least squares.

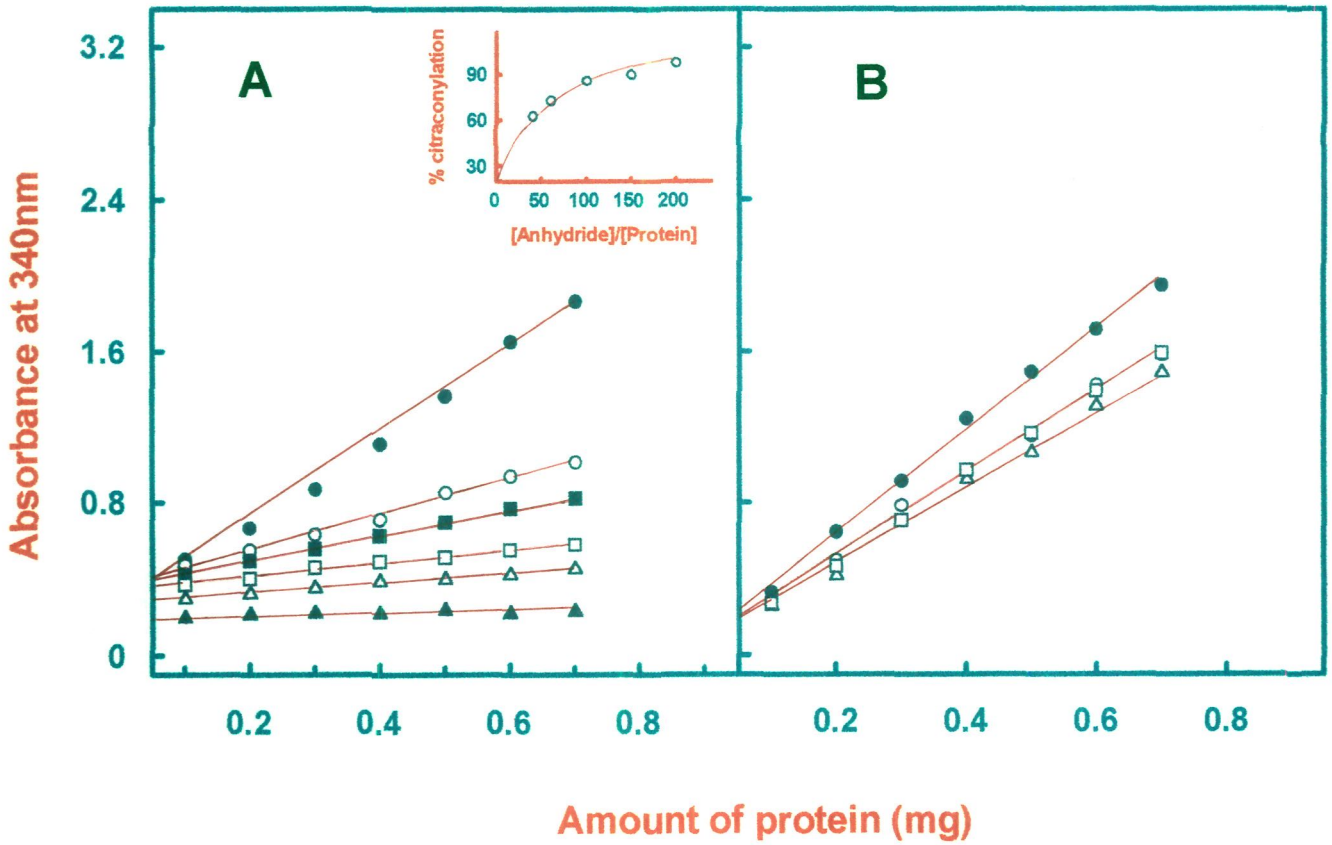


Figure 14. [A] Plots of TNBS color intensity versus protein concentration for BuSA (•); BuSA treated with increasing molar excess of citraconic anhydride viz. (○) 40, (■) 60, (□) 100, (△) 150 and (▲) 200. Inset shows a correlation between extent of citraconylation and the molar ratio of anhydride to protein. [B] Plots of TNBS color intensity versus protein concentration for BuSA (•) and its modified derivatives viz. aBuSA (□), gBuSA (△) and sBuSA (○). Straight lines were drawn by the method of least squares.

results suggests that in all albumins, nearly 80±3% lysine residues are completely accessible to the modifier whereas remaining 20±3% are buried to different extent in the protein interior and thus require higher anhydride concentration for their modification. Based on initial reactivity, BuSA, SSA and RbSA showed differences with HSA and GSA and these differences were eliminated at higher degree of modification. As can be seen from the insets of Figures 10-14A, nearly 90-140 molar excess of anhydride is required to achieve 80% modification of lysine residues of different serum albumins. In order to reversibly block ~80% lysine residues of different serum albumins, the required anhydride concentration was selected from the curves shown in the insets of Figures 10-14A and these albumins were submitted to citraconylation. As desired, the extent of citraconylation in these preparations was found to lie near the expected value of ~80% when checked by TNBS reaction method (Habeeb, 1966). Remaining ~20% buried lysine residues were then modified with a ~100 molar excess of acetic anhydride, succinic anhydride and O-methylisourea after exposing them with 6M guanidine hydrochloride. These preparations were submitted for hydroxylamine treatment (Riordan & Vallee, 1967b) and then dialyzed against 0.05M sodium acetate buffer, pH 3.5 for 96 hours at 10°C to remove the citraconyl groups. Therefore, all these preparations had only internal lysine residues modified. The extent of modification of internal lysine residues in different serum albumins as determined by TNBS reaction method (Figures 10-14B) was found to be 20±3% (Table 3). Taking the total number of amino groups in these albumins as

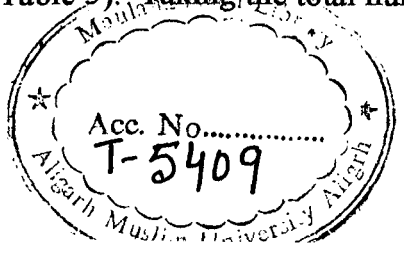


Table 3

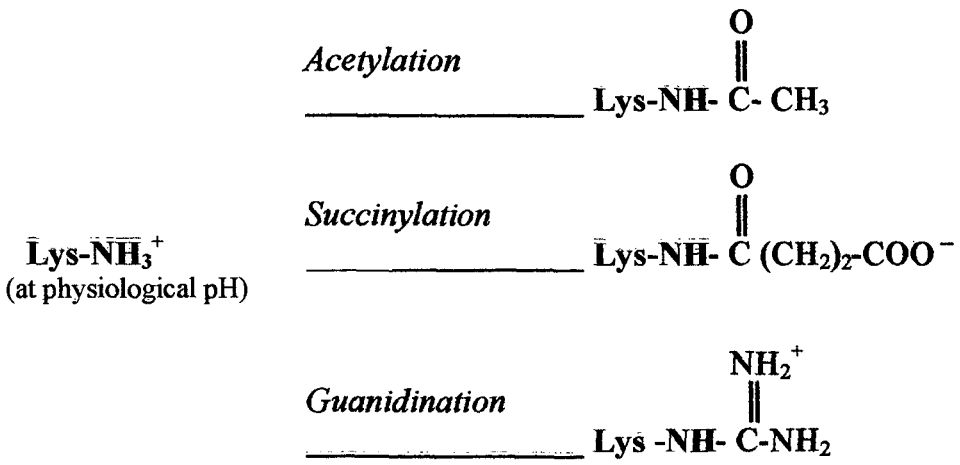
Extent of internal lysine residues modification and its influence on tryptophan (*trp*) fluorescence and mean residue ellipticity (MRE) of different serum albumins.

Albumin derivatives	*ε-NH_2 groups[a] modified (%)*	*trp fluorescence intensity and λ_{max} (nm)*	*MRE_{262nm}[b] (deg. cm^2. $dmol^{-1}$)*	*MRE_{222nm} (deg. cm^2. $dmol^{-1}$)*
HSA	0 (0)	100 (340)	−124	−20010
gHSA	19 (11)	99 (337)	−128	−19980
aHSA	17 (10)	88 (331)	−97	−19890
sHSA	18 (11)	80 (330)	−88	−19870
RbSA	0 (0)	100 (343)	−122	−19620
gRbSA	23 (13)	96 (341)	−124	−19600
aRbSA	20 (12)	81 (334)	−92	−19070
sRbSA	22 (13)	74 (332)	−80	−18640
GSA	0 (0)	100 (342)	−126	−20000
gGSA	19 (11)	97 (340)	−127	−19860
aGSA	21 (13)	82 (333)	−95	−19850
sGSA	18 (11)	75 (333)	−88	−19280
SSA	0 (0)	100 (342)	−126	−20300
gSSA	23 (13)	95 (341)	−126	−20068
aSSA	20 (12)	83 (335)	−98	−20079
sSSA	22 (13)	80 (332)	−89	−20020
BuSA	0 (0)	100 (342)	−120	−19873
gBuSA	23 (13)	96 (340)	−122	−19780
aBuSA	19 (11)	83 (335)	−108	−19560
sBuSA	19 (11)	77 (331)	−100	−19550

[a]Each value of percent modification represents an average of 2-3 independent observations with a precision of 3%. Percent modification was calculated by taking total number of amino groups in different serum albumins as mentioned in the Materials and Methods section. Number of amino groups modified are given in parentheses.

[b]In GSA and BuSA, the MRE values were calculated by taking into account the total number of amino acid residues as 584.

60 or 59 (59 or 58 ε-NH_2 + 1 α-NH_2), the number of lysine residues modified was found to be 10-13 (see Table 3). This was in good agreement to the value reported for BSA (Jonas & Weber, 1970; Mir *et al.*, 1992). Since the same 80% citraconylated preparation of various albumins was modified with three different modifiers after dividing it into three parts, it was assumed that the same set of internal lysine residues was modified in these preparations. Modification of ε-NH_2 group of lysine residues can be viewed as under:



All these preparations (acetylated, succinylated and guanidinated) were found to be homogeneous with respect to size and charge as they eluted in a single symmetrical peak from a Seralose-6B gel filtration column (Figures 15-19) and gave a single band on 8% polyacrylamide gel (insets of Figures 15-19). Since acetylation and succinylation replace the positive charge on lysine residues with zero charge and a negative charge respectively, a slight increase in relative mobility in some of these

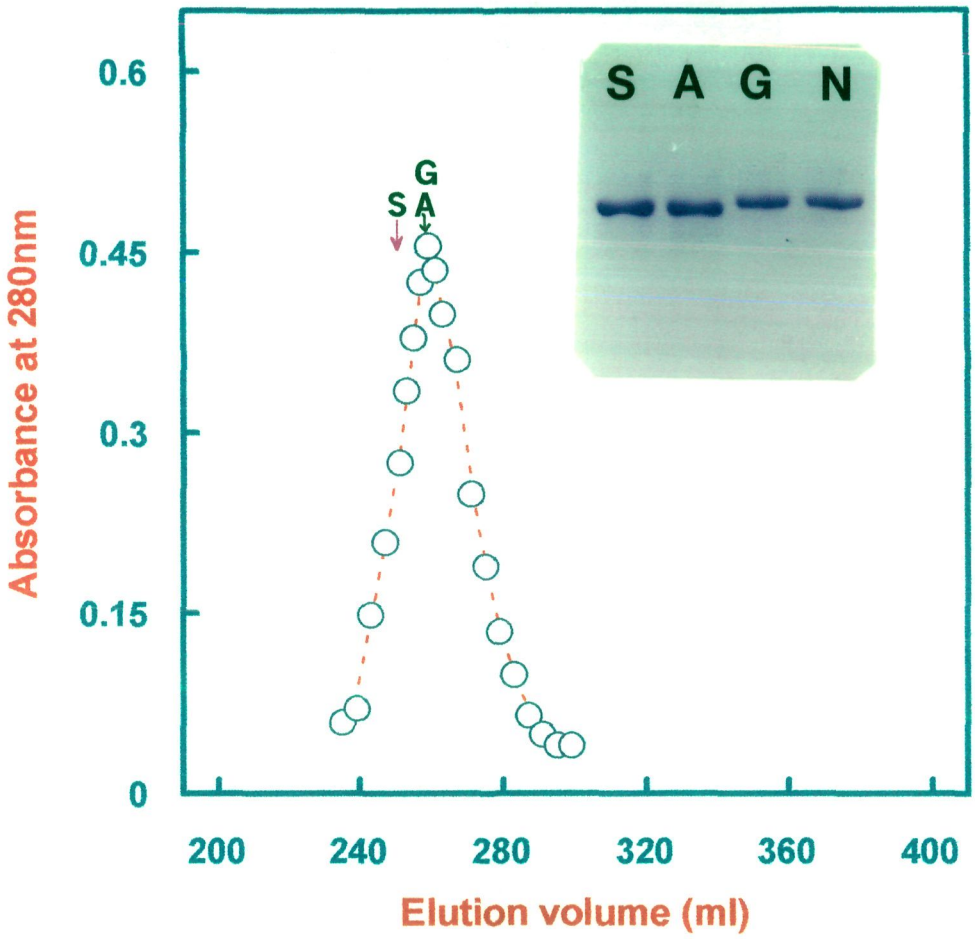


Figure 15. Elution profile of HSA (○) obtained on a Seralose - 6B column (80 x 1.15cm) equilibrated with 0.06 M sodium phosphate buffer, pH 7.4. Letters 'A', 'G' and 'S' indicate the positions of acetylated, guanidinated and succinylated HSA derivatives. Inset shows electrophoretic pattern of native and modified HSA derivatives on 8 % polyacrylamide gel. Same symbols are used for modified derivatives whereas 'N' stands for native HSA.

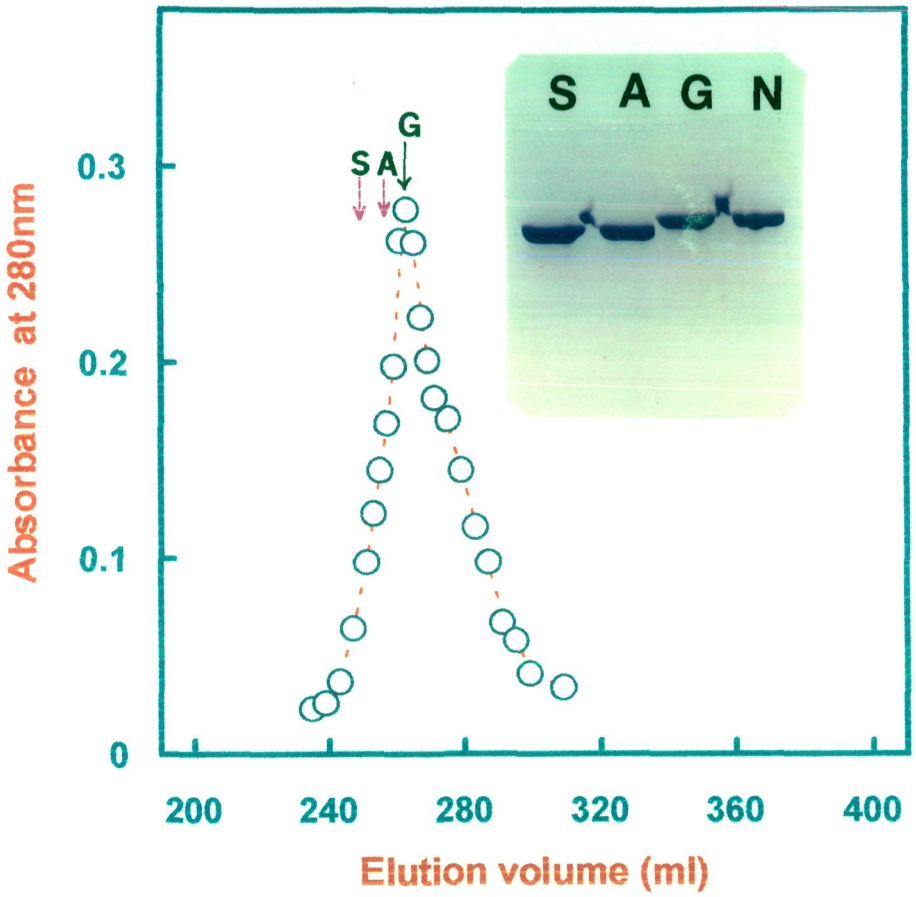


Figure 16. Elution profile of RbSA (○) obtained on a Seralose - 6B column (80 x 1.15 cm) equilibrated with 0.06 M sodium phosphate buffer, pH 7.4. Letters 'A', 'G' and 'S' indicate the positions of acetylated, guanidinated and succinylated RbSA derivatives. Inset shows electrophoretic pattern of native and modified RbSA derivatives on 8 % polyacrylamide gel. Same symbols are used for modified derivatives whereas 'N' stands for native RbSA.

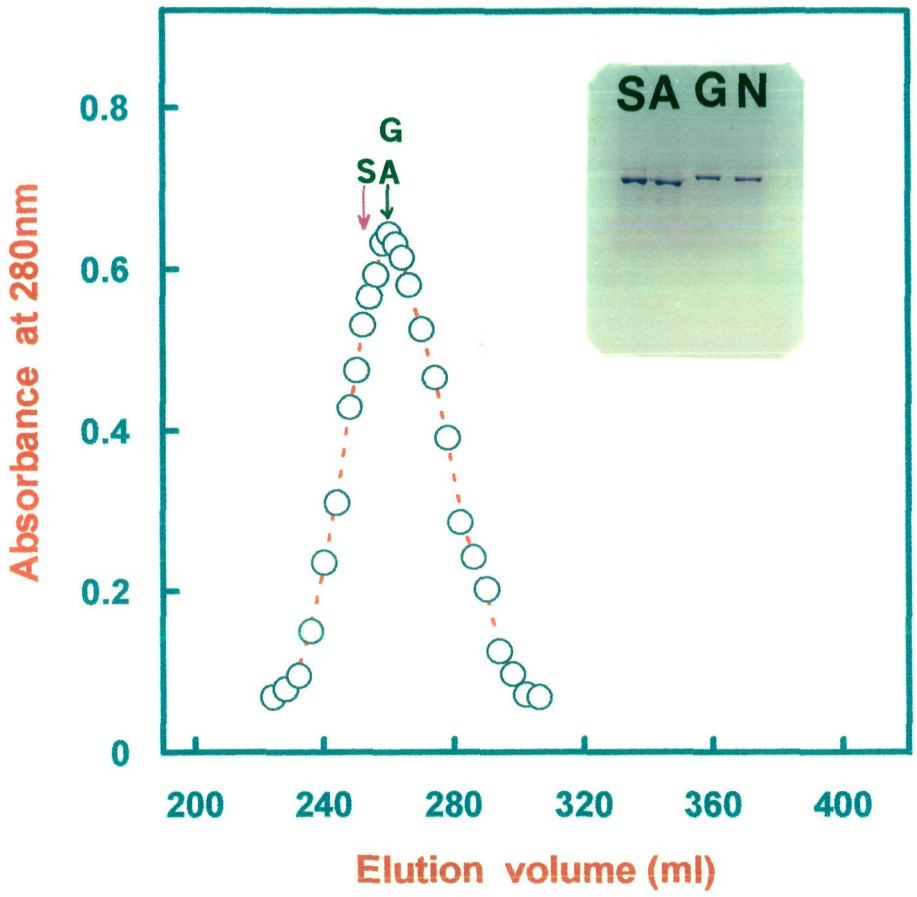


Figure 17. Elution profile of GSA (○) obtained on a Seralose - 6B column (80 x 1.15 cm) equilibrated with 0.06 M sodium phosphate buffer, pH 7.4. Letters 'A', 'G' and 'S' indicate the positions of acetylated, guanidinated and succinylated GSA derivatives. Inset shows electrophoretic pattern of native and modified GSA derivatives on 8 % polyacrylamide gel. Same symbols are used for modified derivatives whereas 'N' stands for native GSA.

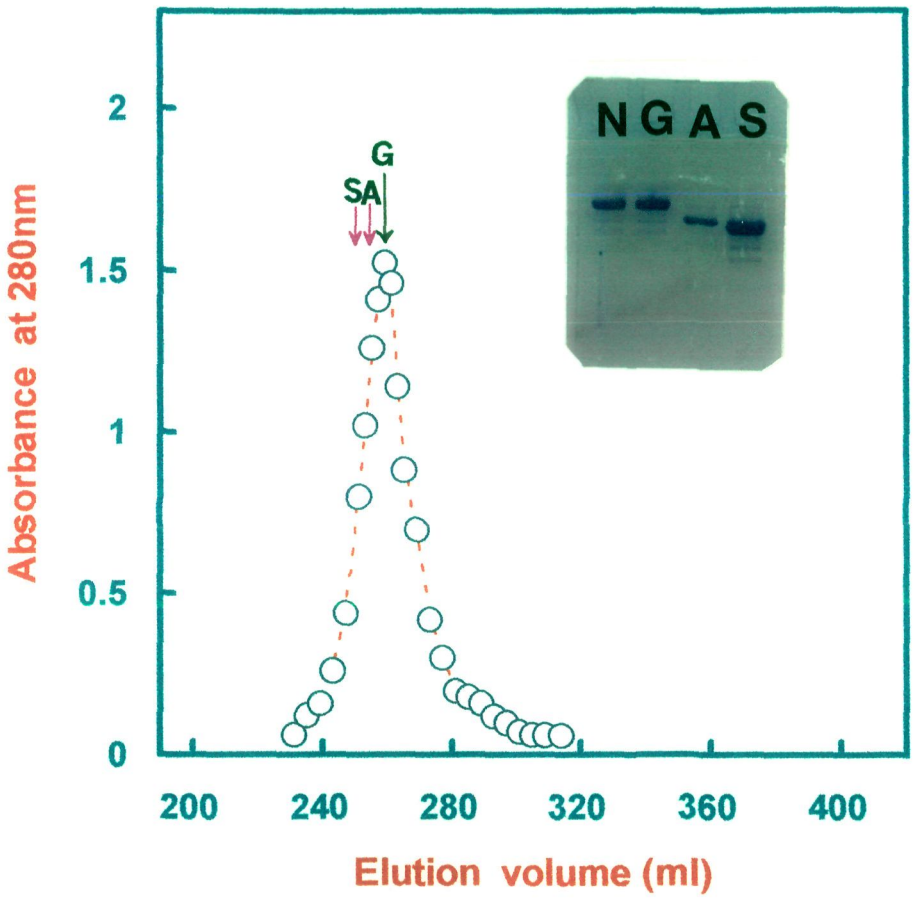


Figure 18. Elution profile of SSA (○) obtained on a Seralose - 6B column (80 x 1.15 cm) equilibrated with 0.06 M sodium phosphate buffer, pH 7.4. Letters 'A', 'G' and 'S' indicate the positions of acetylated, guanidinated and succinylated SSA derivatives. Inset shows electrophoretic pattern of native and modified SSA derivatives on 8 % polyacrylamide gel. Same symbols are used for modified derivatives whereas 'N' stands for native SSA.

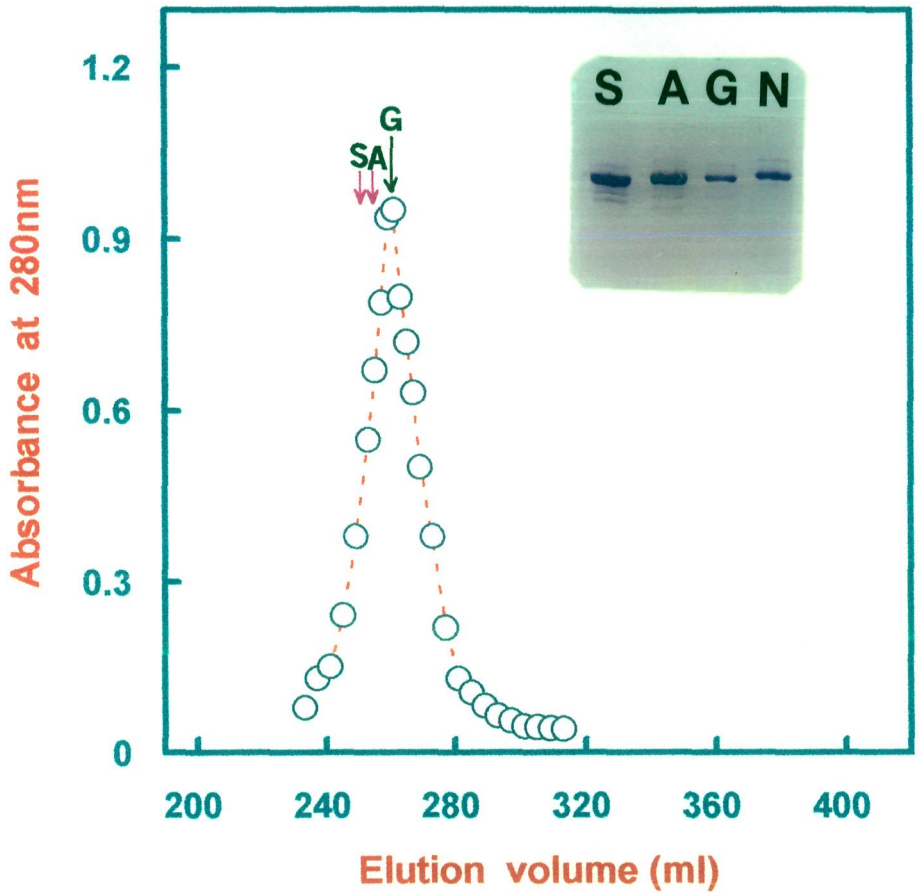


Figure 19. Elution profile of BuSA (○) obtained on a Seralose - 6B column (80 x 1.15 cm) equilibrated with 0.06 M sodium phosphate buffer, pH 7.4. Letters 'A', 'G' and 'S' indicate the positions of acetylated, guanidinated and succinylated BuSA derivatives. Inset shows electrophoretic pattern of native and modified BuSA derivatives on 8 % polyacrylamide gel. Same symbols are used for modified derivatives whereas 'N' stands for native BuSA.

preparations is understandable (insets of Figures 15-19). Guanidinated preparations, on the other hand, moved with the mobility similar to that of native albumin.

Far-UV CD

Figures 20-24A show far UV-CD spectra of various native and modified albumin derivatives obtained at pH 7.4. Spectra were characterized by the presence of two minima at 208 and 222 nm, characteristics of α-helical structure (Sjoholm & Ljungstedt, 1973; Dockal *et al.*, 2000). Acetylation or succinylation of internal lysine residues of different serum albumins resulted in a small decrease in the MRE value being higher in succinylated preparations compared to acetylated derivatives (see Table 3). A maximum decrease of 5% was observed in sRbSA followed by sGSA which showed 3.6% decrease (Table 3). Other serum albumin derivatives did not show any detectable change in the helical content upon acetylation as well as succinylation of internal lysine residues. These results were in accordance with the earlier reports suggesting that the secondary structures are independent and resistant to chemical modification (Batra *et al.*, 1990).

Near - UV CD

Structural changes in the three dimensional conformation of different serum albumins, induced by covalent modification of internal lysine residues were checked by CD measurements in the near-UV region. Figures 20-24B show near-UVCD spectra of

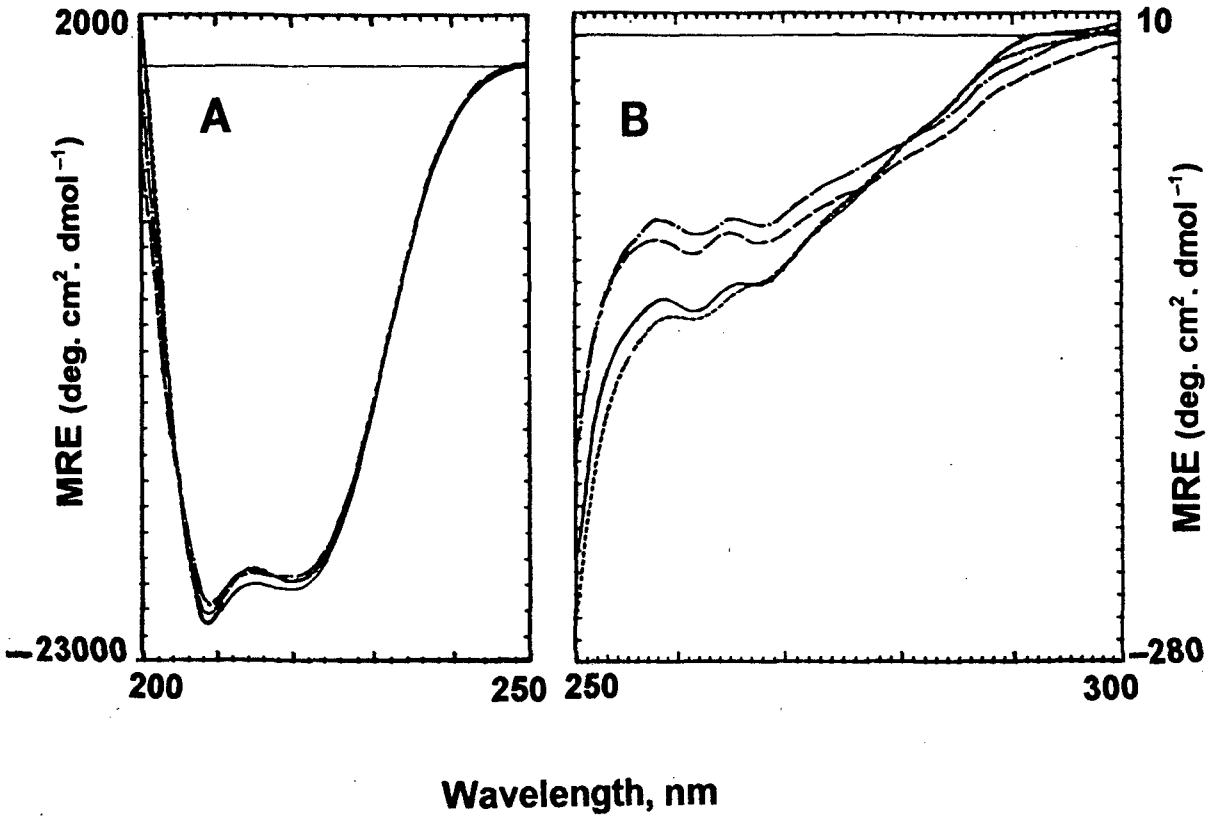


Figure 20. [A] Far-UV and [B] near-UV CD spectra of HSA (—) and its modified derivatives viz. gHSA (---), aHSA (– –) and sHSA (–•–).

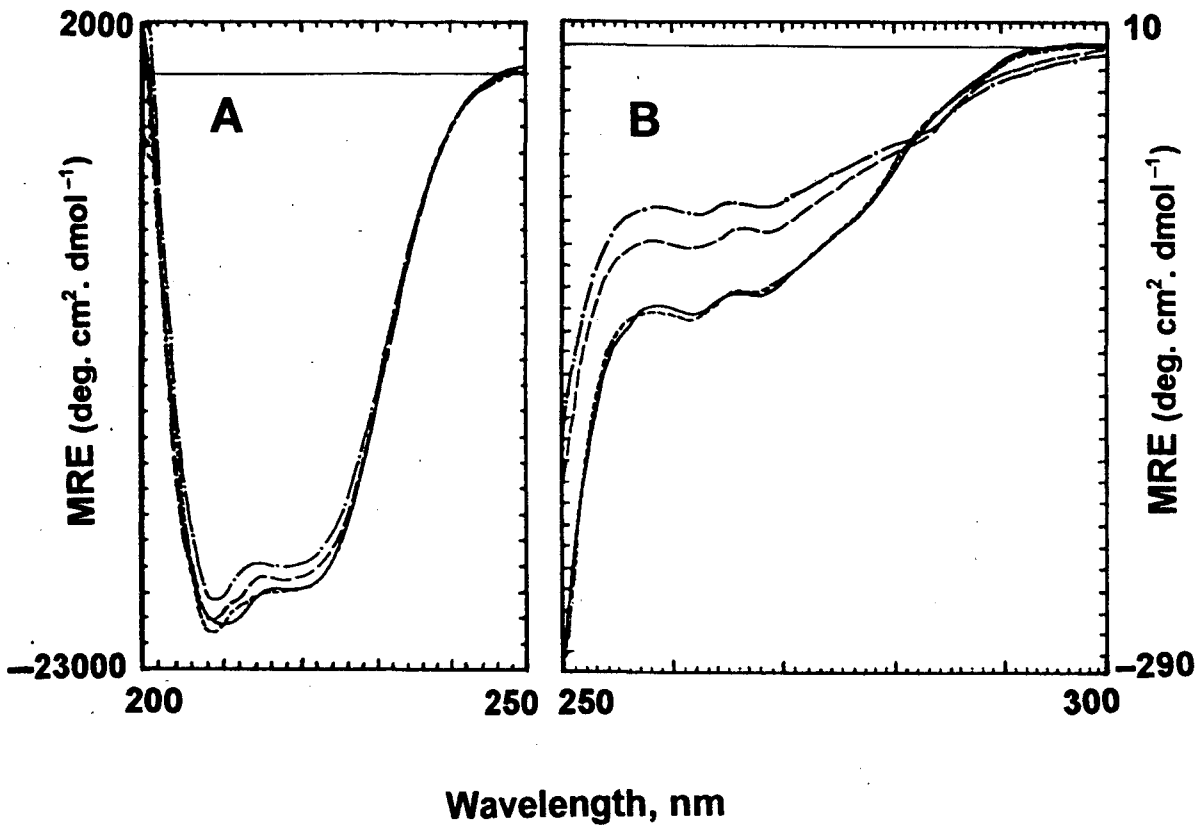


Figure 21. [A] Far-UV and [B] near-UV CD spectra of RbSA (—) and its modified derivatives viz. gRbSA (---), aRbSA (– –) and sRbSA (–•–).

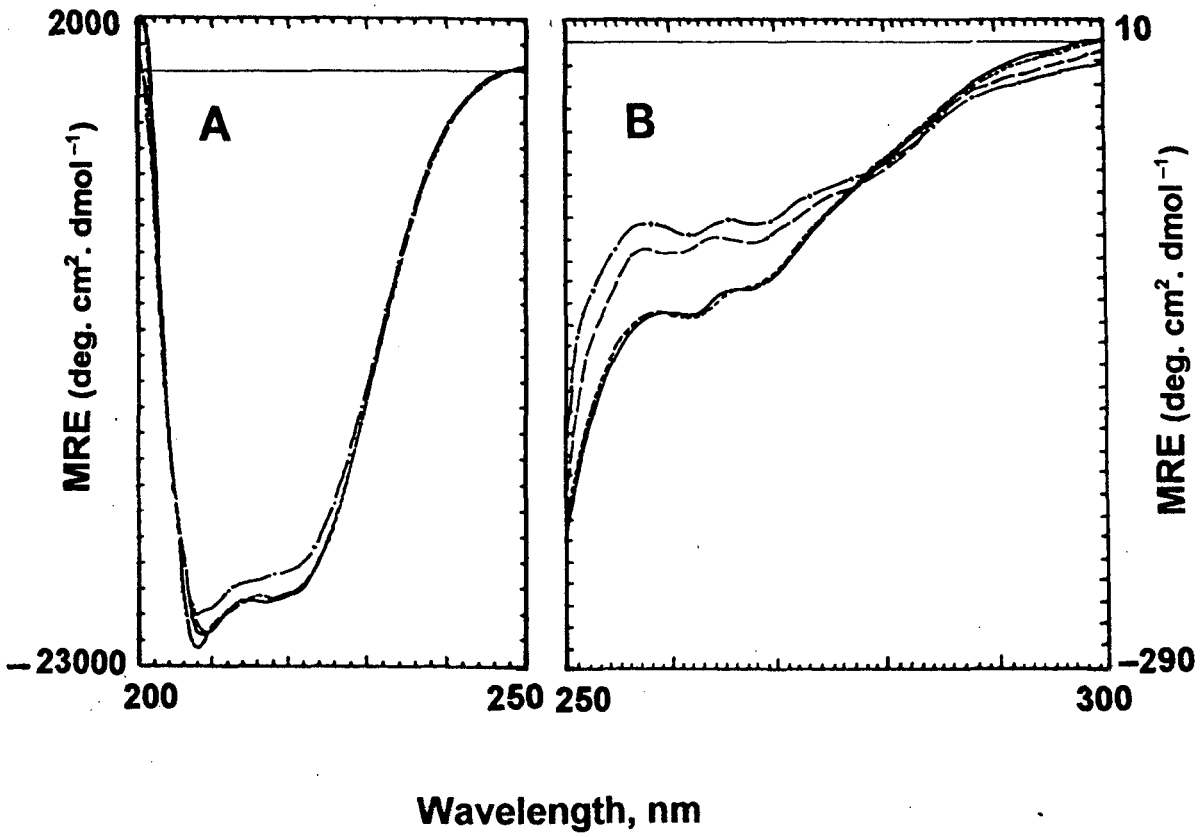


Figure 22. [A] Far-UV and [B] near-UV CD spectra of GSA (—) and its modified derivatives viz. gGSA (---), aGSA (– –) and sGSA (–•–).

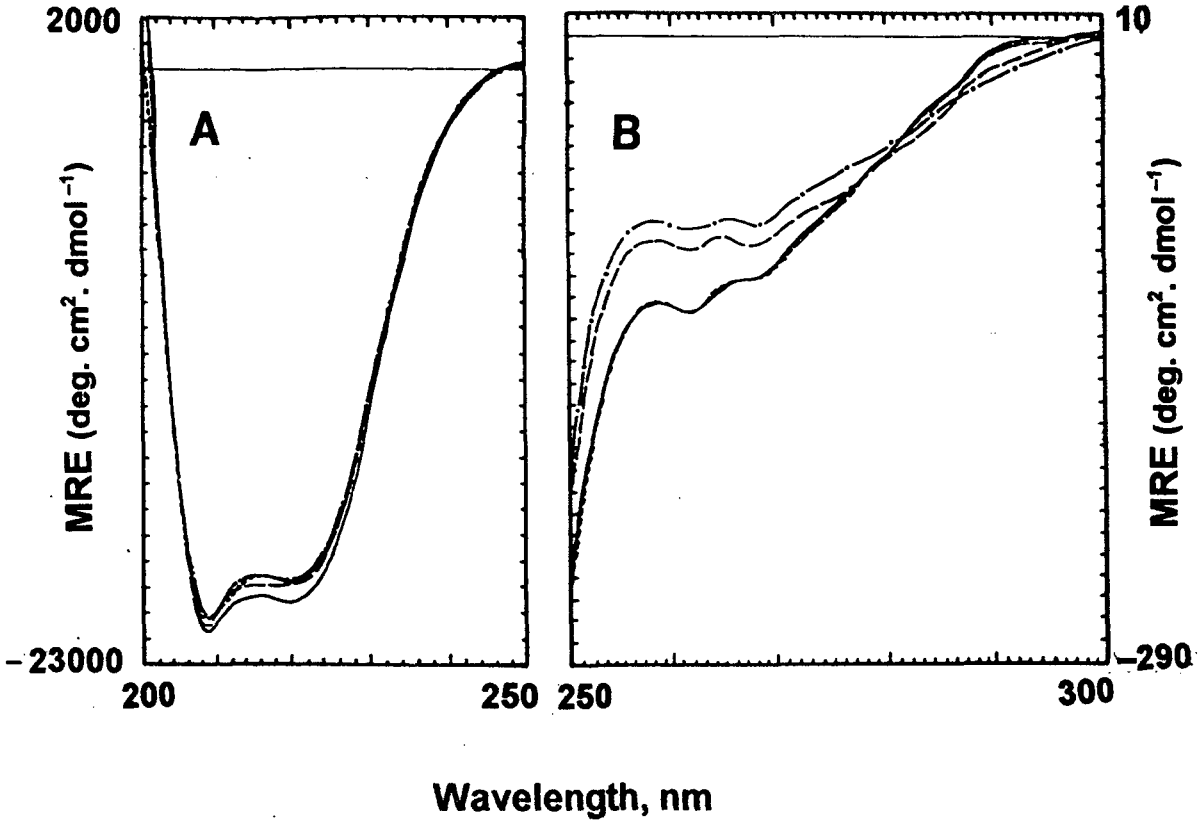


Figure 23. [A] Far-UV and [B] near-UV CD spectra of SSA (—) and its modified derivatives viz. gSSA (---), aSSA (– –) and sSSA (–•–).

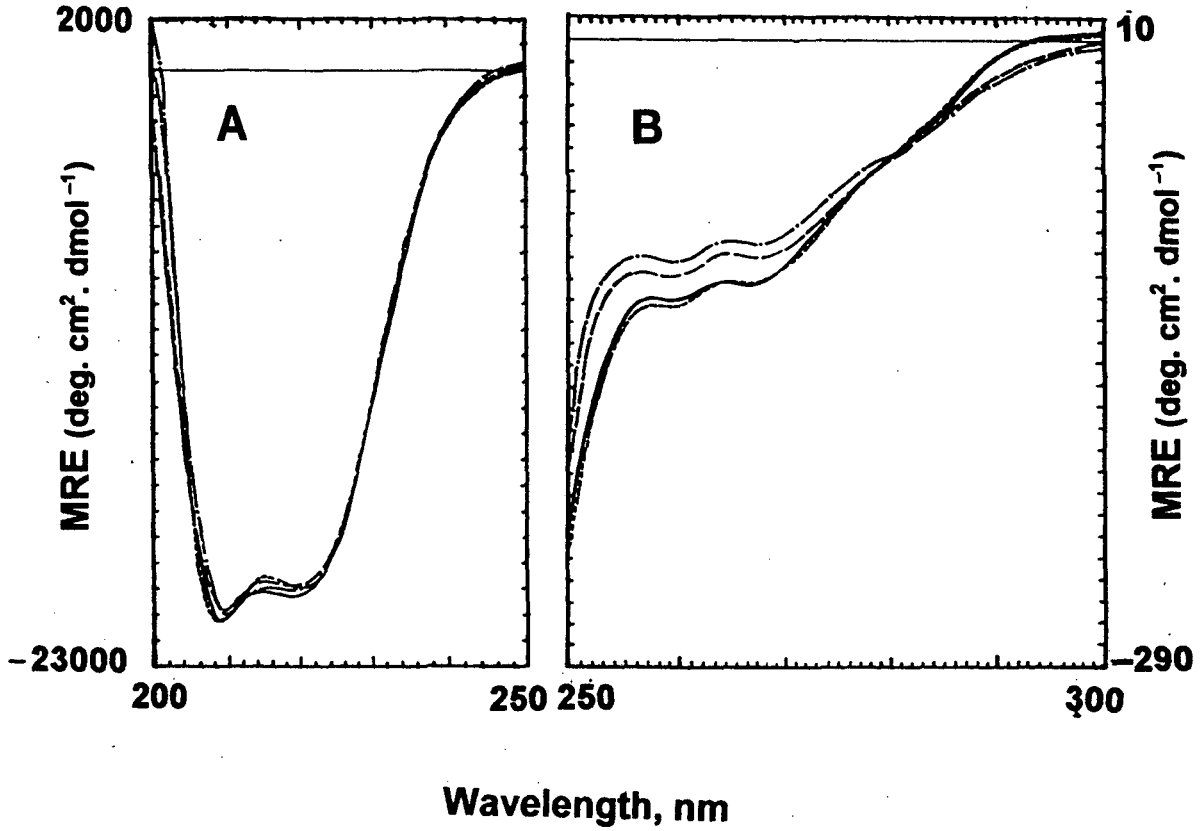


Figure 24. [A] Far-UV and [B] near-UV CD spectra of BuSA (—) and its modified derivatives viz. gBuSA (---), aBuSA (– –) and sBuSA (–•–).

native and modified albumins recorded in the wavelength range of 250-300 nm. As can be seen from these figures, spectra of native albumins show two minima at 262 and 268 nm as well as two shoulders around 277 and 285 nm respectively, characteristics of aromatic chromophores and disulfide bridges. Near-UVCD spectra of different serum albumins were in agreement with earlier observations reported for HSA and BSA (Sjoholm & Ljungstedt, 1973; Sjodin *et al.*, 1977; Dockal *et al.*, 2000). These spectral characteristics in the near-UVCD spectra though retained by all the modified derivatives of different serum albumins, quantitative differences in the MRE values were noticed among these derivatives (Table 3). In acetylated as well as succinylated albumin derivatives of different serum albumins, a decrease in the ellipticity value both at 262 and 268 nm was noticed whereas above 280 nm, the ellipticity value increased slightly indicating the change in the asymmetry of the protein's aromatic amino acids' environment and/or disulfide bridges (Sogami *et al.*, 1982; Muzammil *et al.*, 1999; Dockal *et al.*, 2000). A slight increase in the ellipticity value between 280 and 300 nm in different acetylated and succinylated derivatives can be ascribed to the immobilization of tryptophan side chain in a more hydrophobic environment (Dockal *et al.*, 2000) which was in accordance with the blue shift in the tryptophan emission maxima (as shown in Figure 25) observed with these derivatives. On the other hand, near-UVCD spectra of guanidinated albumin derivatives were more or less similar to the one obtained with native albumins. These results clearly suggested the importance of positive charge on the internal lysine

residues in maintaining the structural integrity of native protein conformation. Similar changes in the ellipticity values have also been reported during pH dependent conformational transition of HSA and BSA (Sogami *et al.*, 1982; Muzammil *et al.*, 1999; Dockal *et al.*, 2000). Therefore, disruption of some intra-molecular salt bridge interactions, steric manifestations due to bulkier acetyl and succinyl groups and charge-charge repulsion, all contribute towards the perturbed tertiary packing in acetylated and succinylated albumin derivatives.

Tryptophan fluorescence

Based on the available amino acid sequence data, nearly all mammalian serum albumins contain one or two tryptophan residues located either in domain II or both in domains I and II respectively (Carter & Ho, 1994). In an earlier study, bilirubin binding site in HSA has been suggested to lie in the close vicinity of tryptophan of domain II at a distance of 27 Å (Sudlow *et al.*, 1976; Berde *et al.*, 1979). Taken together, the involvement of buried lysine residue(s) of BSA in bilirubin-albumin interaction (Mir *et al.*, 1992) and the location of bilirubin binding site in the close vicinity of tryptophan (Berde *et al.*, 1979) it seems necessary to investigate the changes in the microenvironment around tryptophan residue(s) after the modification of buried lysine residues of these albumins. Changes in the environment around tryptophan residue(s) can be precisely detected from the fluorescence properties of modified albumins by using an excitation wavelength of 295 nm where

only tryptophan is excited and contribution from other aromatic chromophores is negligible (Schmid, 1997). Figure 25 (A-E) shows tryptophan fluorescence spectra of different serum albumins and their modified derivatives. Qualitatively similar fluorescence spectra were observed with different serum albumins and their derivatives. However, quantitative differences in the fluorescence intensity and emission maxima were noticed (see Table 3). As can be seen from Figure 25 and Table 3, both quantum yield and emission maxima affected to an appreciable extent by acetylation as well as succinylation of internal lysine residues in all the serum albumins under investigation, whereas guanidination did not affect these parameters to a significant extent. Decrease in fluorescence intensity (12-26%) and shift in the emission maxima towards shorter wavelength by about 9-11 nm in acetylated and succinylated derivatives of all the albumins suggested that few acetyl or succinyl groups were incorporated on to those critical lysine residues which seem to be located in the close vicinity of tryptophan and thus changed the microenvironment. Since the extent of blue shift and decrease in fluorescence intensity observed with HSA (containing a lone tryptophan-214 in domain II) upon acetylation or succinylation were more or less similar to those observed with the same derivatives of other albumins, it can be said that change in the microenvironment was mainly confined to the tryptophan present in domain II in these albumins. These observations suggest that some of the internal lysine residues make a significant contribution to the binding site architecture for Sudlow's Site I, a specialized cavity in subdomain IIA which

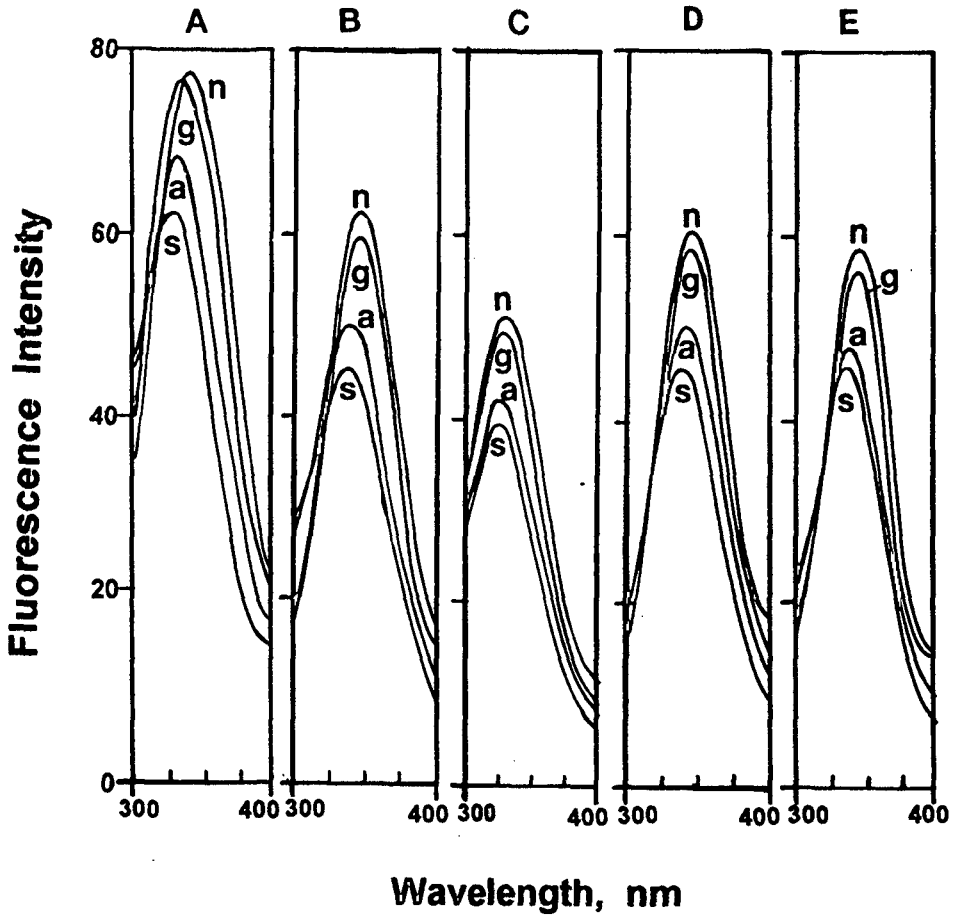


Figure 25. Tryptophan fluorescence spectra of (A) HSA, (B) RbSA, (C) GSA, (D) SSA and (E) BuSA. Letters **n**, **g**, **a** and **s** represent native, acetylated, guanidinated and succinylated albumin derivatives respectively. The spectra were recorded after exciting the protein at 295 nm. Slits were 2 and 3 nm for excitation and emission respectively.

accommodates at least one tryptophan residue (Sudlow *et al.*, 1976; Carter & Ho, 1994).

A significant (12-26%) decrease in tryptophan fluorescence observed in different albumins upon acetylation or succinylation can be ascribed to the restriction / alteration of the mobility of indole side chain of tryptophan due to altered environment induced by the attached acetyl or succinyl groups. Marked blue shift in the emission maxima of different acetylated or succinylated albumins indicated changes in the dielectric microenvironment around tryptophan towards more hydrophobic or apolar (Sjoholm & Ljungstedt, 1973; Dockal *et al.*, 2000). This seems to be understandable as positive charge of lysine residues is abolished in acetylated preparations and substituted with a negative charge in succinylated derivatives and the presence of acetyl or succinyl groups will certainly increase the hydrophobicity of the binding pocket. These results also suggest the involvement of buried lysine residues in salt bridge(s) formation with oppositely charged groups.

Molecular size

Interdomain associations involving hydrophobic as well as salt bridge interactions play important role in the maintenance of native three dimensional conformation of albumin (Dockal *et al.*, 2000). Two such salt bridges, involving Lys-190 and Lys-205 in association with Glu-425 and Glu-465 respectively have been found in a high-resolution crystal structure of HSA (Carter & Ho, 1994). Covalent blocking of few

such lysine residues in albumin by acetylation or succinylation would abolish some of the intramolecular and interdomain interactions and thus may affect native protein conformation and/or molecular size (Ansari *et al.*, 1975). The effect may be more significant in the case of succinyl group incorporation (which replaces a positive charge on lysine residues with a negative charge) due to charge-charge repulsion as well as steric hindrance caused by succinyl groups (Ansari *et al.*, 1975; Kidwai *et al.*, 1976). This can be clearly seen from the values of Stokes radius for different albumins and their modified derivatives obtained from analytical gel filtration data (see Table 4). HSA and SSA have little higher molecular size, with a Stokes radius of ~3.56 nm each compared to RbSA which is smaller having a Stokes radius of 3.35 nm. Although, all serum albumins showed increase in Stokes radius upon succinylation, the effect of acetylation on the Stokes radius was seen in some cases (Table 4). The increase in Stokes radius of different serum albumins upon succinylation of their buried lysine residues was in agreement with an earlier report on BSA (Mir *et al.*, 1992).

Bilirubin binding studies

Binding of bilirubin to different serum albumins and their modified derivatives was studied by fluorescence quench titration, absorption and CD spectroscopy. Using fluorescence quench titration method and by fitting the data into Scatchard plots (Figures 26-30), we found comparable values of association constant, K_a (1.42×10^7 -

Table 4

Gel filtration data of different serum albumins and their modified derivatives obtained on a Seralose-6B column (80 x 1.15cm) at pH 7.4.

Albumin derivatives	*Elution volume (ml)*	$(-\log K_{av})^{1/2}$	$erfc^{-1}\ K_d$	*Stokes radius*[a] (nm)
HSA	259	0.4269	0.2829	3.56
gHSA	259	0.4269	0.2829	3.56
aHSA	259	0.4269	0.2829	3.56
sHSA	257	0.4362	0.2873	3.64
RbSA	263	0.4123	0.2596	3.35
gRbSA	263	0.4123	0.2596	3.35
aRbSA	260	0.4233	0.2731	3.49
sRbSA	258	0.4305	0.2832	3.58
GSA	260	0.4233	0.2731	3.49
gGSA	261	0.4196	0.2689	3.45
aGSA	260	0.4233	0.2731	3.49
sGSA	258	0.4305	0.2832	3.58
SSA	259	0.4269	0.2829	3.56
gSSA	259	0.4269	0.2829	3.56
aSSA	257	0.4362	0.2873	3.64
sSSA	256	0.4378	0.2921	3.67
BuSA	261	0.4196	0.2689	3.45
gBuSA	261	0.4196	0.2689	3.45
aBuSA	259	0.4269	0.2829	3.56
sBuSA	258	0.4305	0.2832	3.58

[a]Values of Stokes radius represent a mean of two data sets analyzed according to equation (3) and (4) respectively as mentioned in the 'Materials and Methods' section.

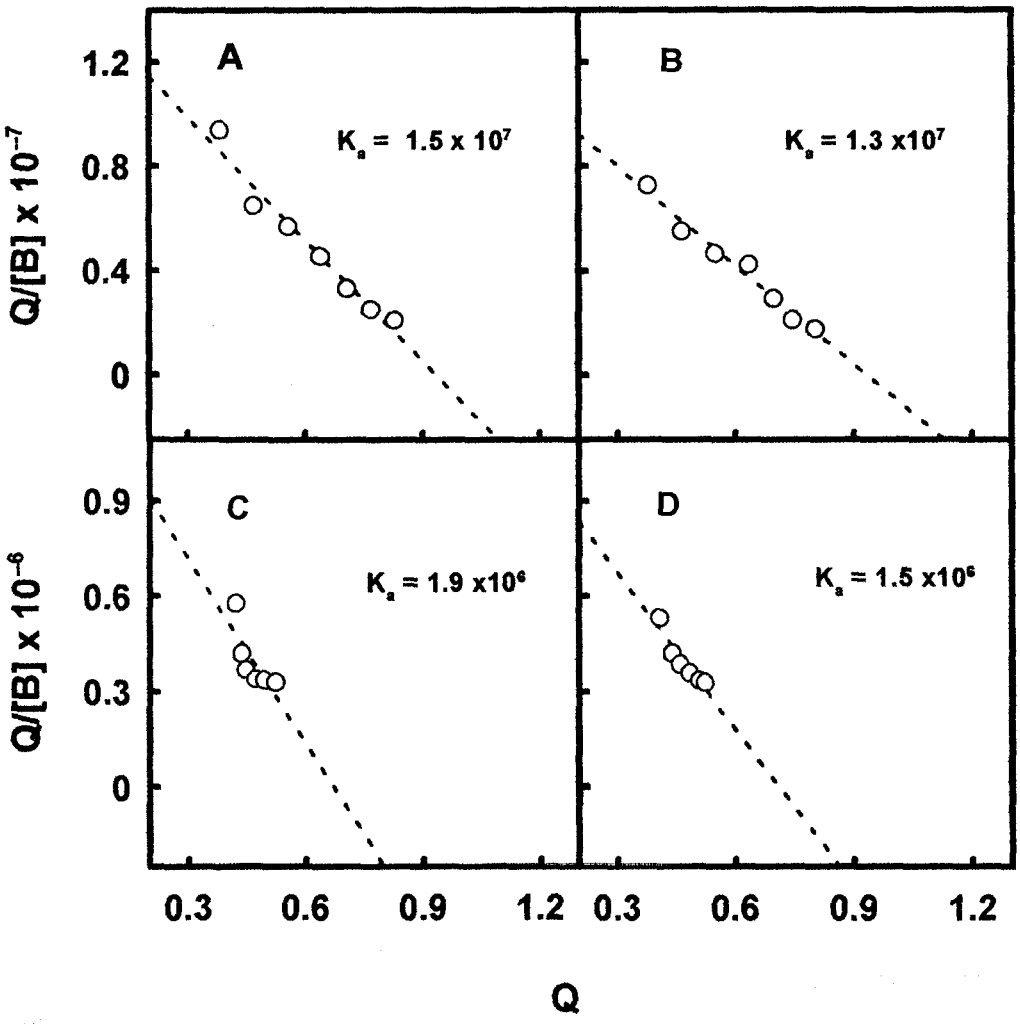


Figure 26. Scatchard plots for the interaction of bilirubin with HSA (A) and its modified derivatives viz. gHSA (B), aHSA (C) and sHSA (D).

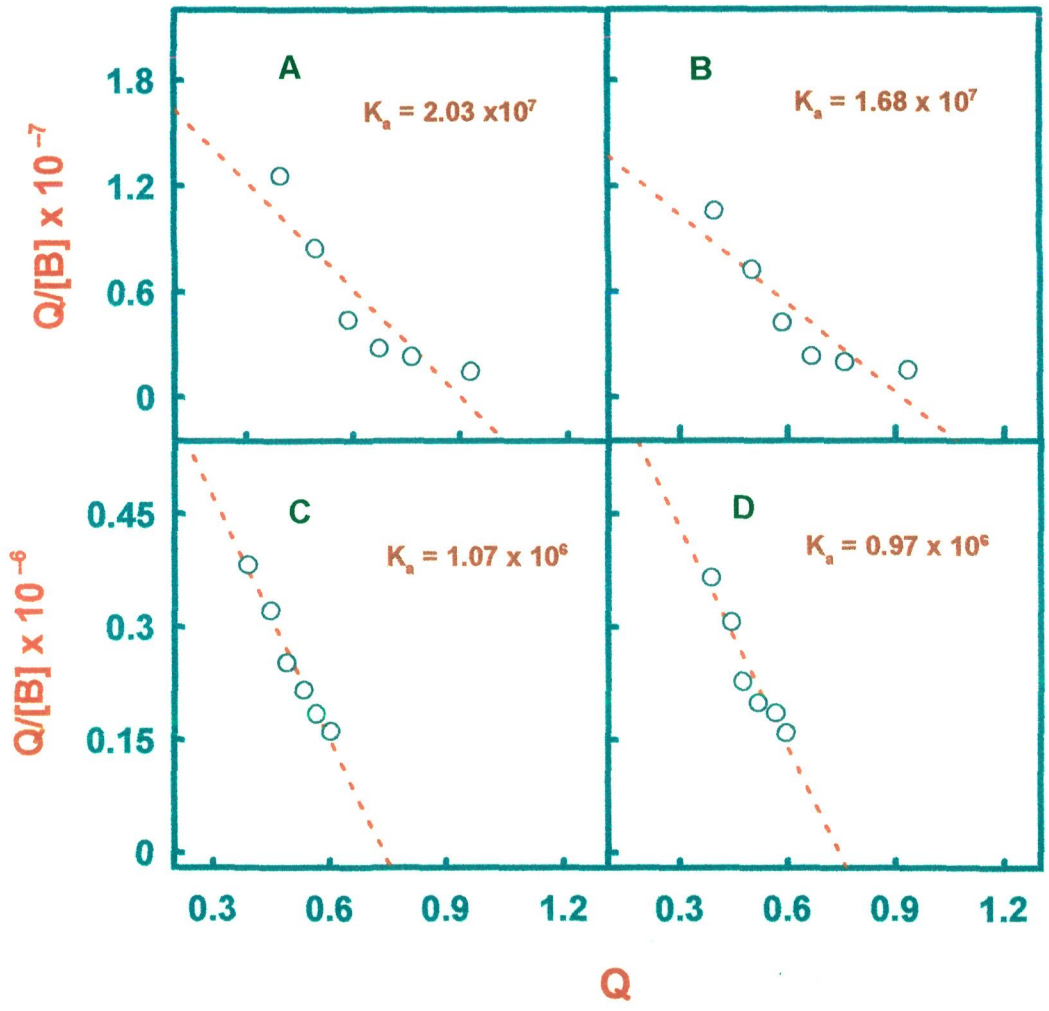


Figure 27. Scatchard plots for the interaction of bilirubin with RbSA (A) and its modified derivatives viz. gRbSA (B), aRbSA (C) and sRbSA (D).

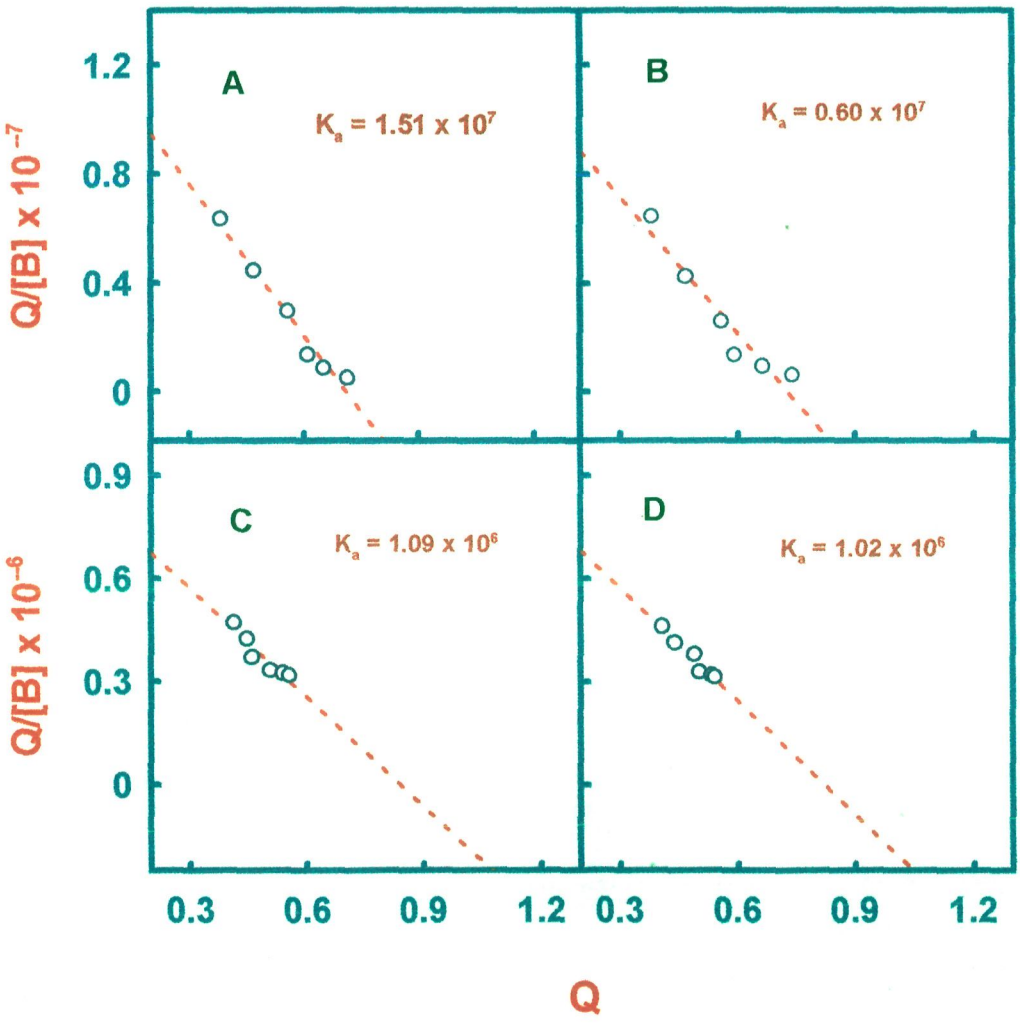


Figure 28. Scatchard plots for the interaction of bilirubin with GSA (A) and its modified derivatives viz. gGSA (B), aGSA (C) and sGSA (D).

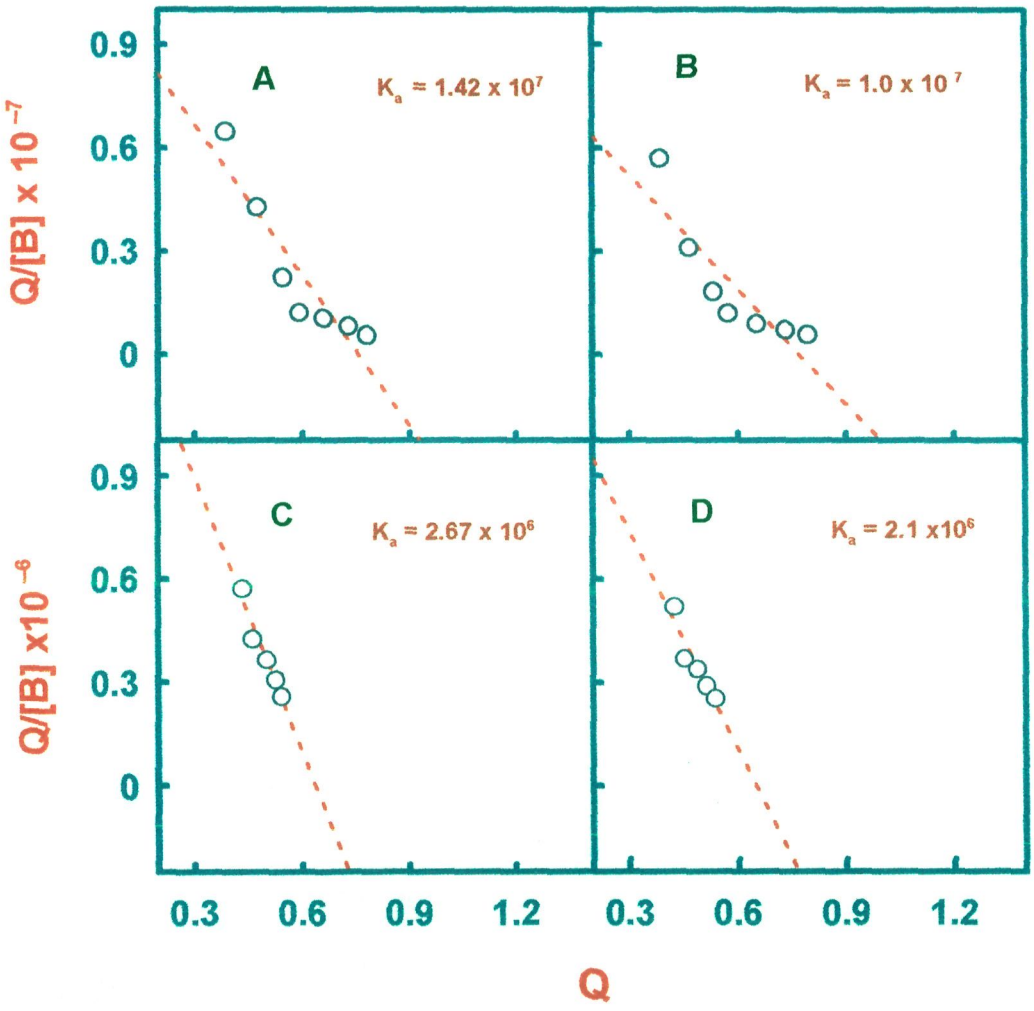


Figure 29. Scatchard plots for the interaction of bilirubin with SSA (A) and its modified derivatives viz. gSSA (B), aSSA (C) and sSSA (D).

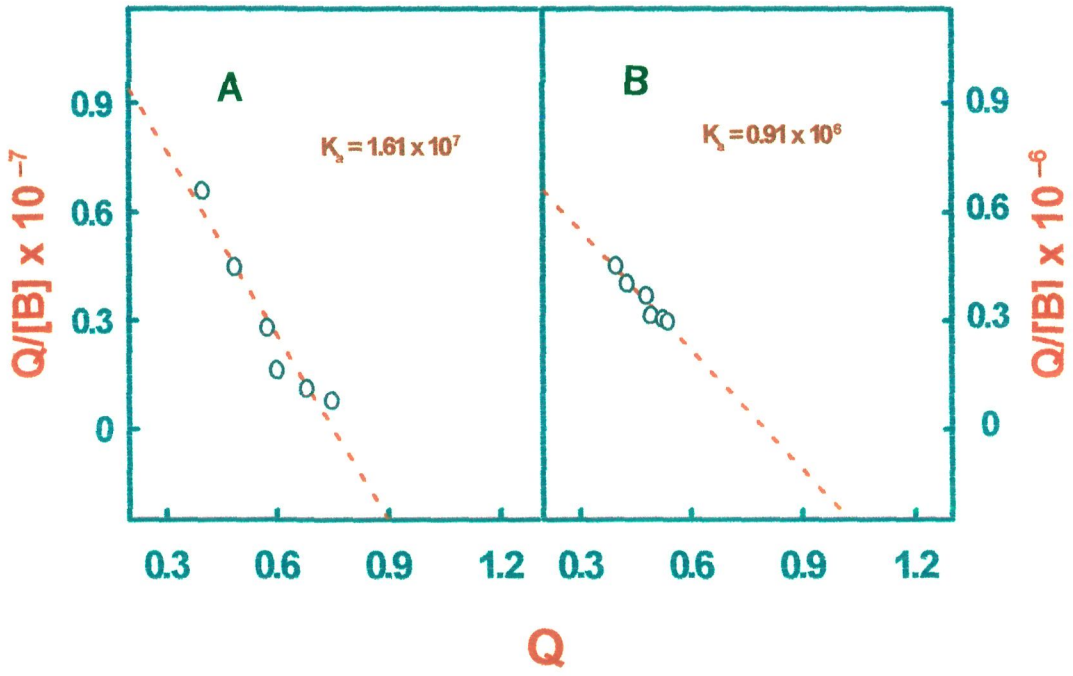


Figure 30. Scatchard plots for the interaction of bilirubin with BuSA (A) and its modified derivative viz. aBuSA (B).

2.03 x10^7 lit. mol^{-1}) of bilirubin for the primary binding site on different serum albumins as shown in Table 5. These values were in agreement with the previously published values of 1.56x10^7 and 1.57x10^7 lit. mol^{-1} reported for HSA and BSA respectively (Berde *et al.*, 1979; Mir *et al.*, 1992). The value of K_a decreased significantly (>5 fold) upon acetylation or succinylation of internal lysine residues of different serum albumins being much higher (~21 fold) in RbSA (see Table 5). On the other hand, guanidinated albumin derivatives gave more or less similar values of K_a as obtained with native albumins. In an earlier report, severe decrease in K_a value of bilirubin binding has also been shown upon acetylation or succinylation of internal lysine residues of BSA (Mir *et al.*, 1992). The decreased affinity of acetylated / succinylated albumin derivatives for bilirubin can be attributed to the abolishment of positive charge on critical lysine residues of these albumins.

Free or unbound bilirubin gave the absorption spectrum in the wavelength range 380-550 nm with a maxima (λ_{max}) around 440 nm. The absorption maxima shifted towards higher wavelength (red shift) and absorbance increased significantly when albumin was added to the bilirubin solution. Both increase in absorption and red shift in the absorption maxima were indicative of the pigment binding to albumin (Harmatz & Blauer, 1975; Jacobsen & Brodersen, 1983; Tayyab *et al.*, 1995). Figures 31-35 show absorption spectra of bilirubin complexed with various native albumins and their covalent derivatives. Values of λ_{max} as well as molar absorption coefficient at λ_{max} of various complexes of bilirubin with albumins and their modified derivatives are

Table 5

Visible range spectral data of bilirubin (10μM) bound to different native albumins and their modified derivatives (20μM) measured in 0.06M sodium phosphate buffer, pH 8.0, ionic strength 0.15.

Albumin derivatives	*Molar absorption[a] coefficient (ϵ x 10^{-5})*	*K_a (lit. mol^{-1})*	*Molar ellipticities and position of CD band maxima and minima (nm) in parentheses.[b]*	
HSA	0.685 (468)	1.50 x10^7	30.73 (460)	–24.12 (409)
gHSA	0.652 (467)	1.30 x10^7	26.00 (460)	–21.40 (409)
aHSA	0.563 (456)	1.90 x10^6	19.11 (462)	–14.21 (411)
sHSA	0.533 (454)	1.50 x10^6	13.12 (454)	–11.15 (409)
RbSA	0.721 (464)	2.03 x10^7	33.80 (458)	–32.00 (413)
gRbSA	0.695 (563)	1.68 x10^7	32.26 (461)	–31.16 (416)
aRbSA	0.560 (452)	1.07 x10^6	17.00 (454)	–14.04 (412)
sRbSA	0.546 (450)	0.97 x10^6	13.20 (466)	–09.16 (414)
GSA	0.725 (472)	1.51 x10^7	—	–36.26 (426) –31.00 (464)
gGSA	0.699 (468)	0.60 x10^7	—	–30.40 (428)
aGSA	0.560 (460)	1.09 x10^6	12.40 (444)	–13.00 (409)
sGSA	0.503 (456)	1.02 x10^6	12.00 (458)	–08.00 (400)
SSA	0.657 (466)	1.42 x10^7	—	–38.20 (472)
gSSA	0.619 (466)	1.00 x10^7	—	–25.00 (414) –31.60 (430)
aSSA	0.506 (456)	2.67 x10^6	16.10 (464)	–18.30 (409)
sSSA	0.452 (451)	2.10 x10^6	22.30 (466)	–12.00 (411)
BuSA	0.645 (468)	1.61 x10^7	—	–36.10 (454)
gBuSA	0.605 (464)	nd	—	–34.20 (450)
aBuSA	0.510 (456)	0.91 x10^6	10.70 (486)	–31.40 (433)
sBuSA	0.464 (448)	nd	19.00 (470)	–24.10 (411)

[a]Values of molar absorption coefficient (ϵ) were calculated at λ_{max} with respect to native albumin-bilirubin complexes and are expressed as ϵ x 10^{-5} M^{-1} cm^{-1}. The concentration of bilirubin and albumin were 10μM and 20μM respectively. Values of λ_{max} are given in parentheses.

[b]Values of molar ellipticity were calculated with respect to the total bilirubin present in the solution and are expressed as [θ] x 10^{-4} (deg. cm^2. $dmol^{-1}$).

nd: not determined

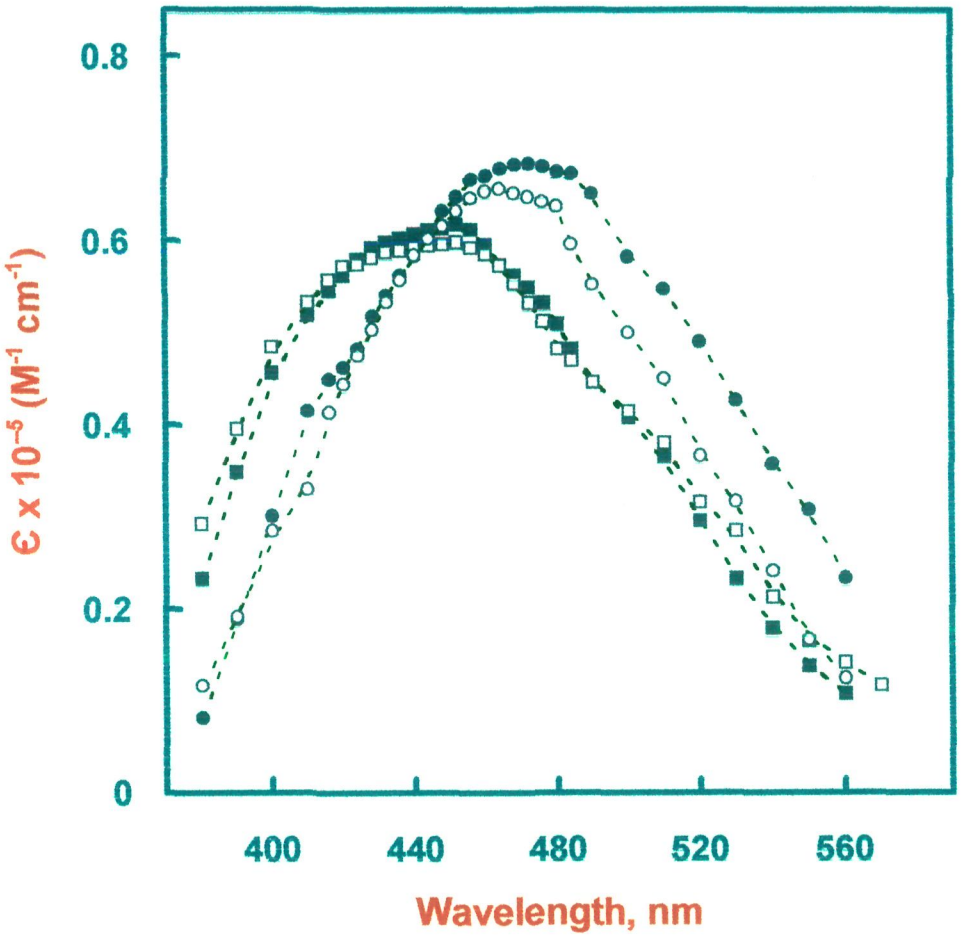


Figure 31. Visible absorption spectra of bilirubin complexed with HSA (•) and its modified derivatives viz. gHSA (○), aHSA (■) and sHSA (□).

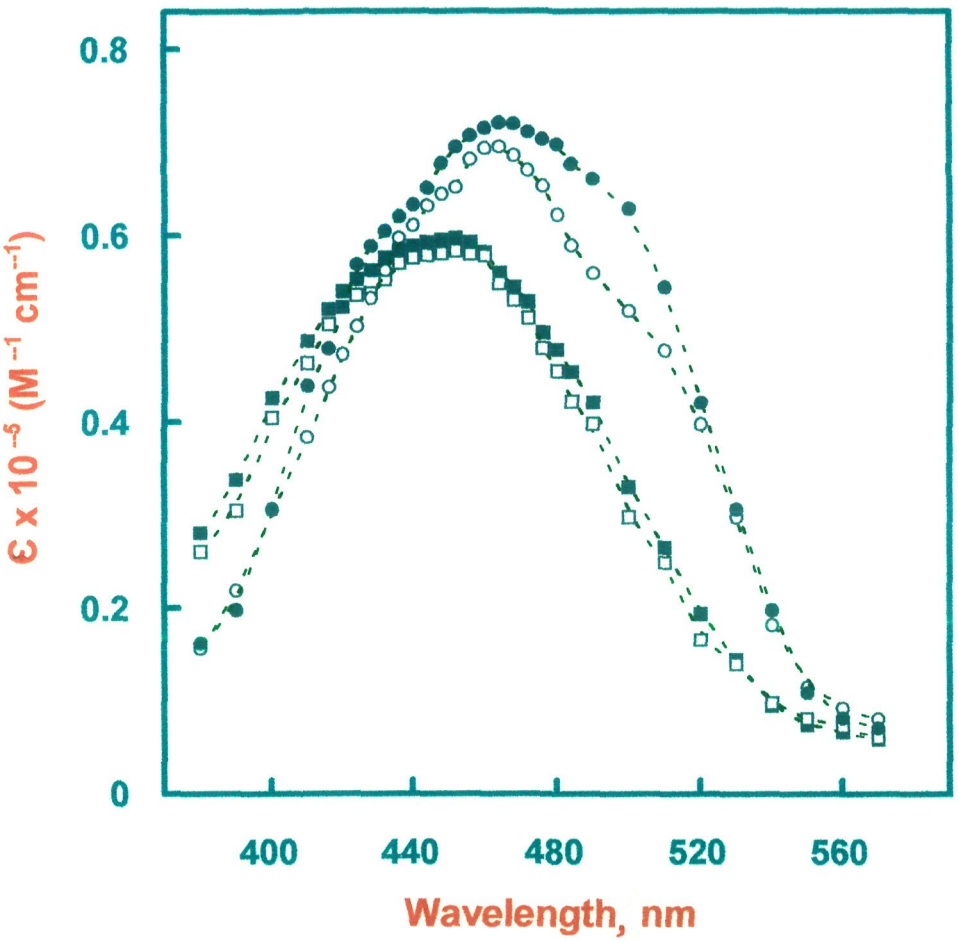


Figure 32. Visible absorption spectra of bilirubin complexed with RbSA (•) and its modified derivatives viz. gRbSA (○), aRbSA (■) and sRbSA (□).

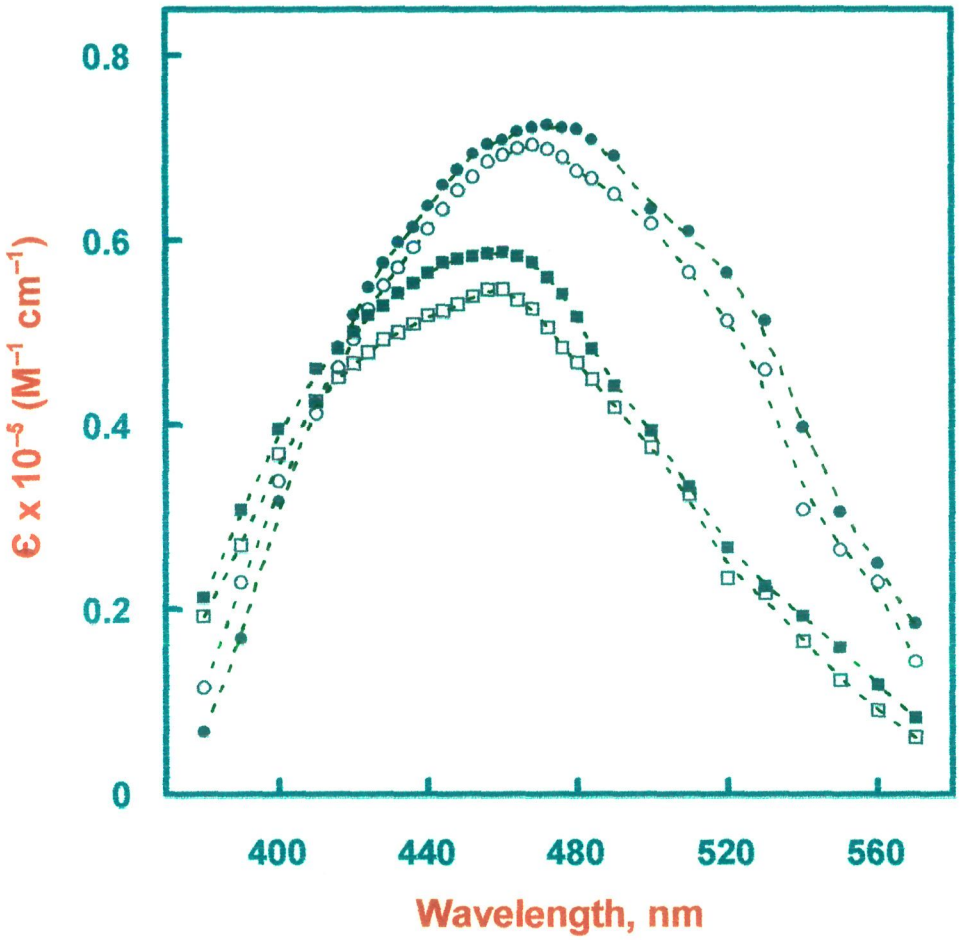


Figure 33. Visible absorption spectra of bilirubin complexed with GSA (•) and its modified derivatives viz. gGSA (○), aGSA (■) and sGSA (□).

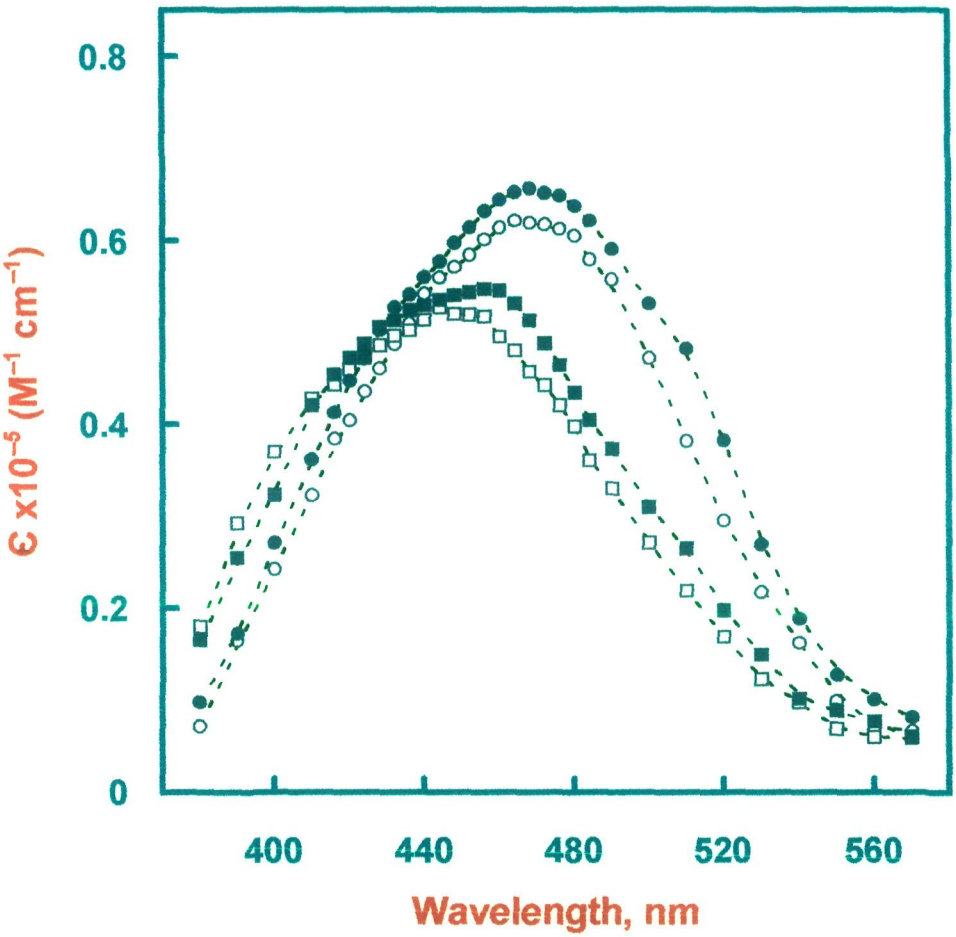


Figure 34. Visible absorption spectra of bilirubin complexed with SSA (•) and its modified derivatives viz. gSSA (○), aSSA (■) and sSSA (□).

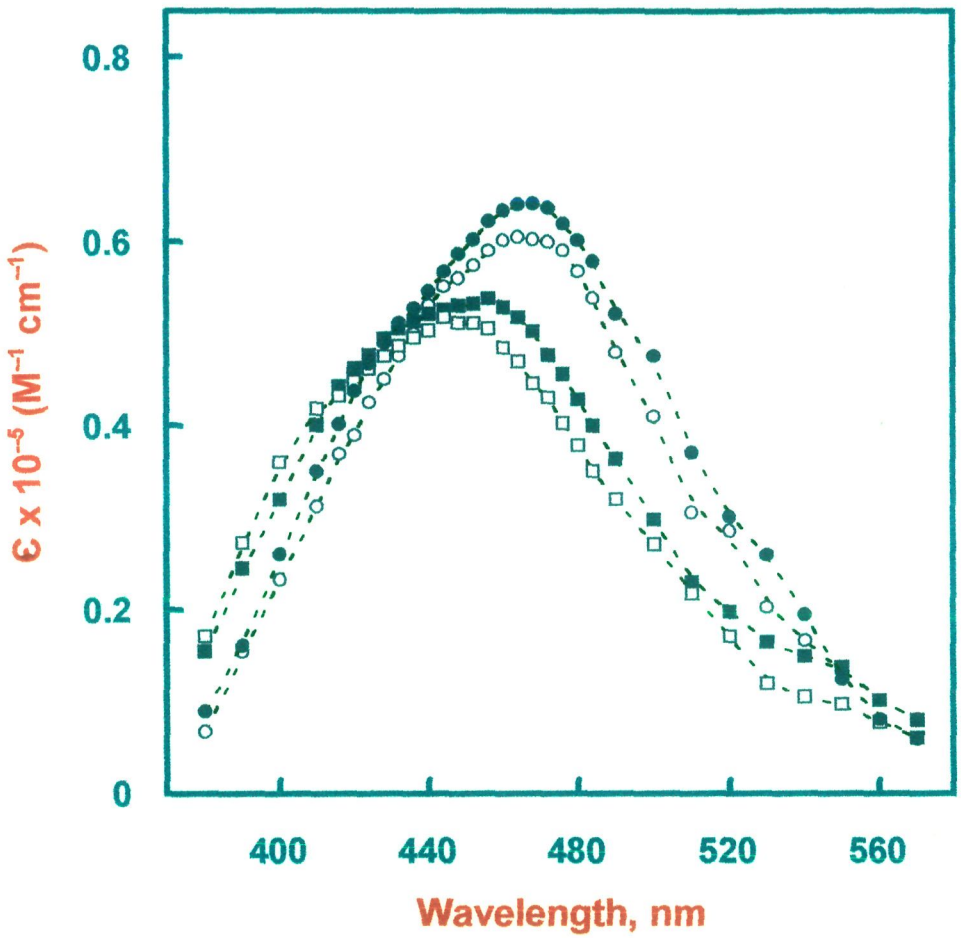


Figure 35. Visible absorption spectra of bilirubin complexed with BuSA (•) and its modified derivatives viz. gBuSA (○), aBuSA (■) and sBuSA (□).

given in Table 5. As is evident from Figures 31-35 and Table 5, all serum albumins produced a strong red shift of about 24-32 nm in the absorption maxima of bilirubin upon binding which was in agreement with earlier observations (Harmatz & Blauer, 1975; Jacobsen, 1977; Berde *et al.*, 1979; Tayyab & Qasim, 1987). The complexes of bilirubin with guanidinated derivatives of these albumins showed somewhat similar characteristics (with little variation) as observed with native albumin-bilirubin complexes. On the other hand, complexes of bilirubin with both acetylated and succinylated derivatives showed a red shift of about 12-20 nm and 8-16 nm respectively. Decrease in the extent of red shift as well as molar absorption coefficient (see Table 5) of the complexes of bilirubin with acetylated and succinylated derivatives of different albumins suggested the role of positive charge on internal lysine residues of these albumins in bilirubin-albumin interaction. This was in agreement with previous reports showing the involvement of electrostatic interactions in bilirubin binding to albumin (Jacobsen, 1977; Roosdorp *et al.*, 1977).

In view of the two half-domain model of primary bilirubin binding site on albumin in which each dipyrrole unit of the pigment binds to separate subdomains of albumin making a dihedral angle and thus leading to exciton splitting which results in changes in the absorption spectrum of bilirubin (Jacobsen & Brodersen, 1983; Lightner *et al.*, 1986; 1988), the alterations in absorption spectrum of bilirubin complexed with acetylated or succinylated albumin derivatives compared to that obtained with native or guanidinated albumins can be ascribed to the shifting of

dihedral angle and thereby the exciton splitting among the two chromophores which brought about significant changes in the conformation of the bound pigment. Similar changes have also been noticed on lowering the pH of bilirubin-albumin solution (Harmatz & Blauer, 1975; Jacobsen & Brodersen, 1983).

Figures 36-40 show the effect of internal lysine modification of serum albumins on the CD spectrum of bilirubin bound to high affinity site in these albumins. As can be seen from Figures 36 and 37, curve **n**, complexes of bilirubin with native HSA and RbSA showed bisignate CD spectra characterized by a positive CD Cotton effect (CDCE) at higher wavelength and negative CDCE at shorter wavelength. These observations were similar to those reported earlier (Harmatz & Blauer, 1975; Lightner *et al.*, 1986; 1988) suggesting that the pigment binds to these albumins in a folded conformation with positive chirality. Acetylation or succinylation of internal lysine residues in these albumins, though did not change the sign order and shape of the bisignate CD spectra but severely decreased the intensity of both positive and negative CDCEs (Figures 36 and 37, curves **a** and **s** and Table 5). In acetylated and succinylated RbSAs, the band maxima were also slightly shifted from their actual positions (Table 5). Nearly 50% (or more) reduction in the original intensity of CD band maxima and minima were noticed in both HSA and RbSA upon acetylation and succinylation of internal lysine residues whereas guanidination resulted in small changes in the CDCEs' intensity (Figures 36 and 37, curve **g**). The altered CD spectra of bilirubin bound to acetylated and succinylated albumins was in agreement with

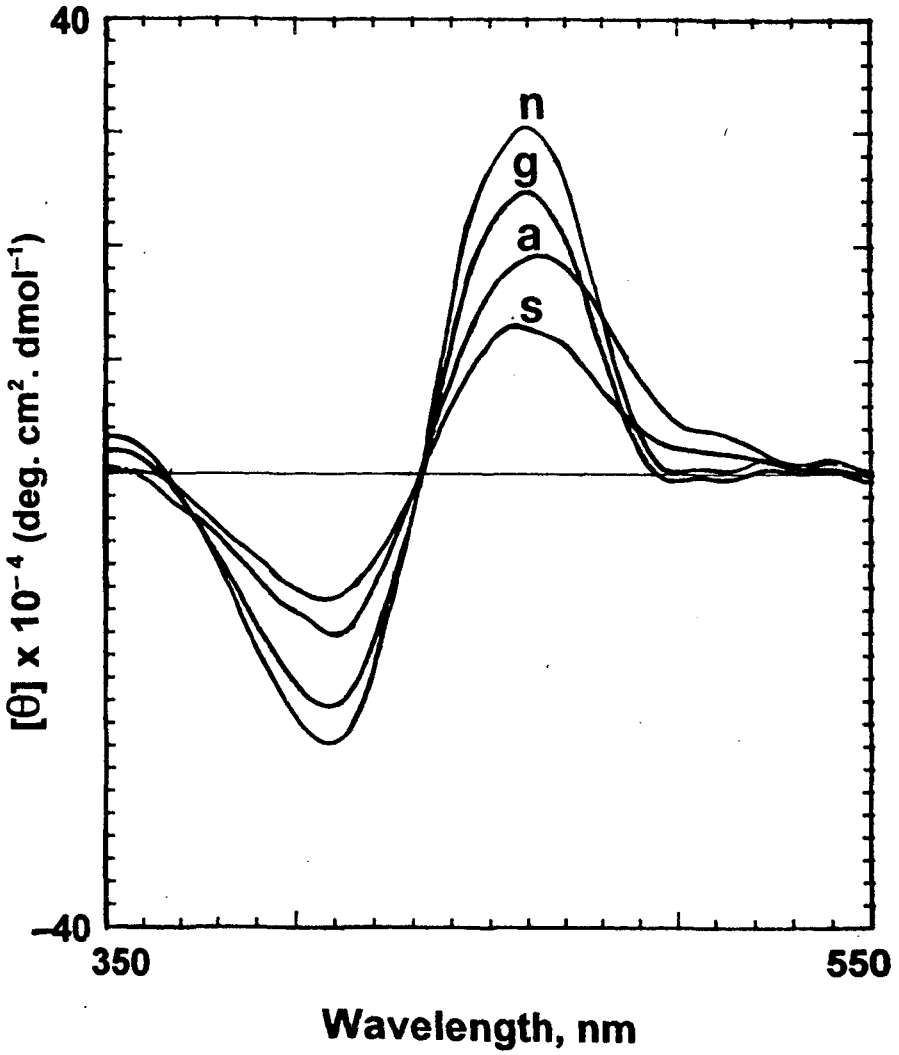


Figure 36. CD spectra of bilirubin (10μM) complexed with HSA and its modified derivatives (20μM). Letters **n, g, a** and **s** stand for native, guanidinated, acetylated and succinylated HSA derivatives respectively.

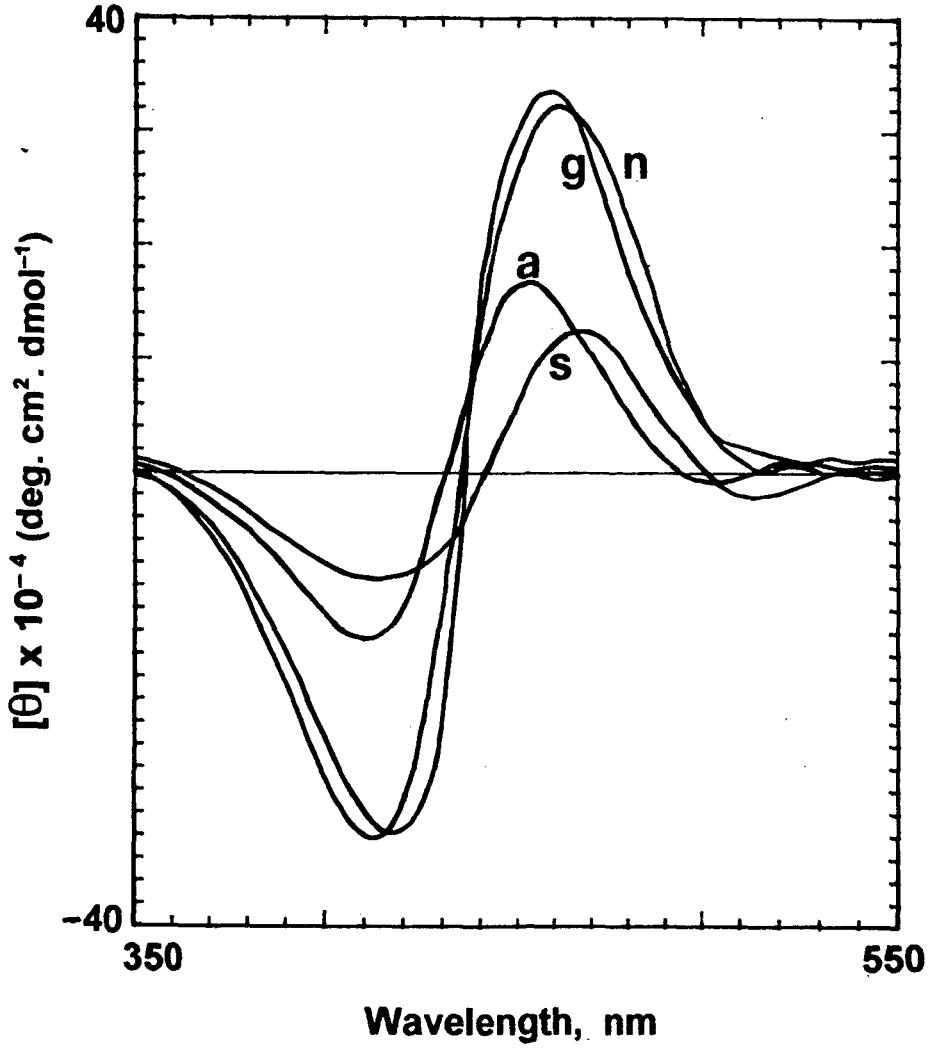


Figure 37. CD spectra of bilirubin (10μM) complexed with RbSA and its modified derivatives (20μM). Letters **n, g, a** and **s** stand for native, guanidinated, acetylated and succinylated RbSA derivatives respectively.

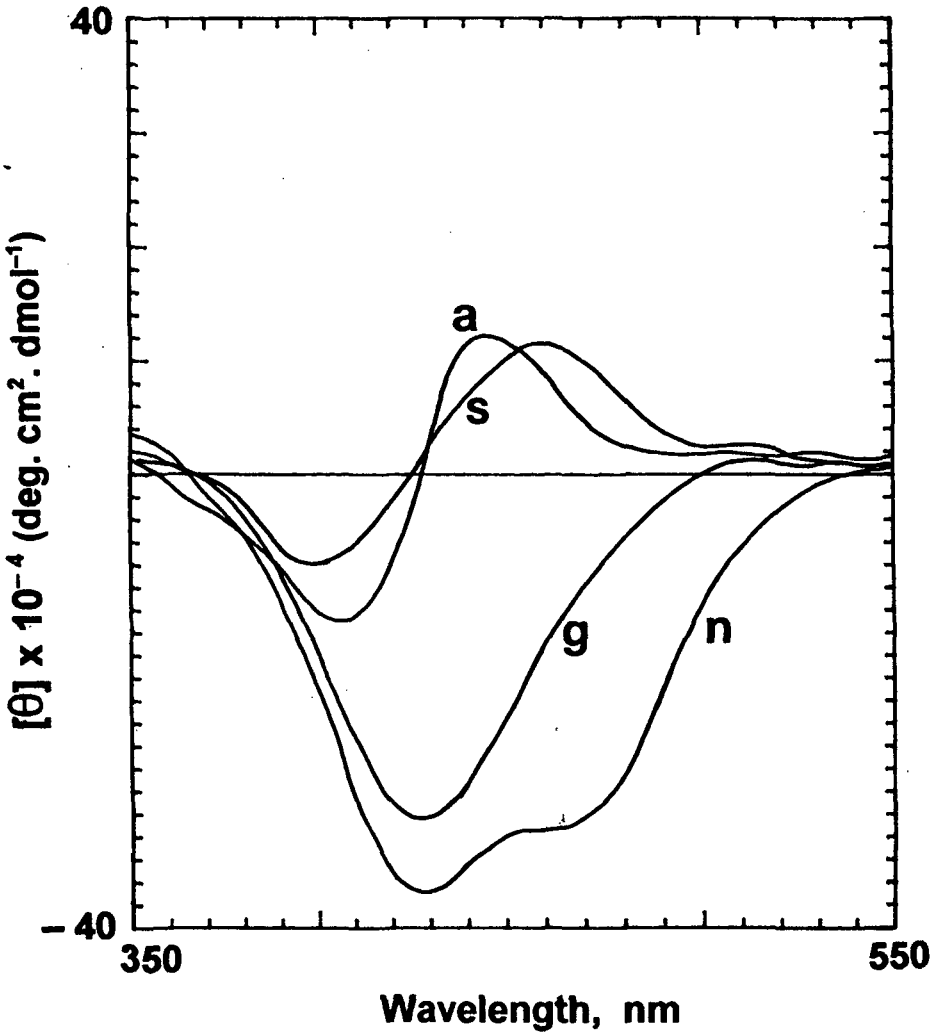


Figure 38. CD spectra of bilirubin (10μM) complexed with GSA and its modified derivatives (20μM). Letters **n, g, a** and **s** stand for native, guanidinated, acetylated and succinylated GSA derivatives respectively.

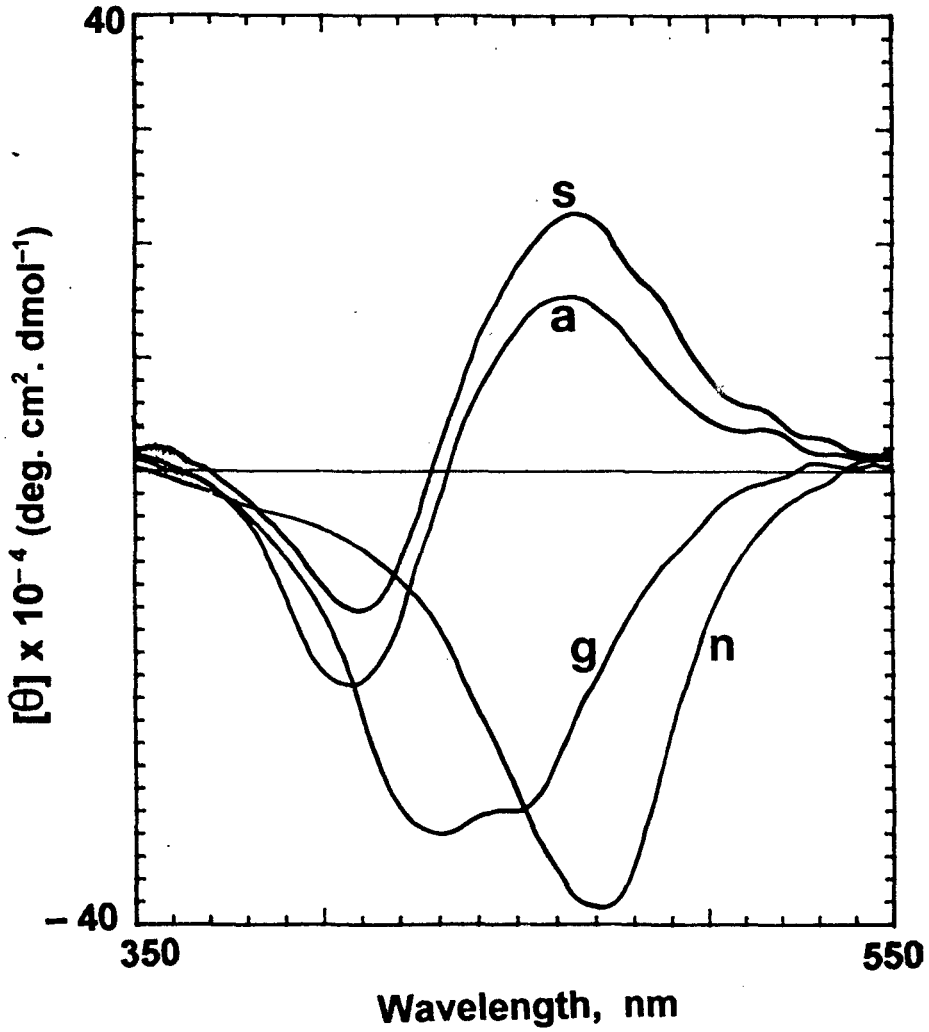


Figure 39. CD spectra of bilirubin (10μM) complexed with SSA and its modified derivatives (20μM). Letters **n, g, a** and **s** stand for native, guanidinated, acetylated and succinylated SSA derivatives respectively.

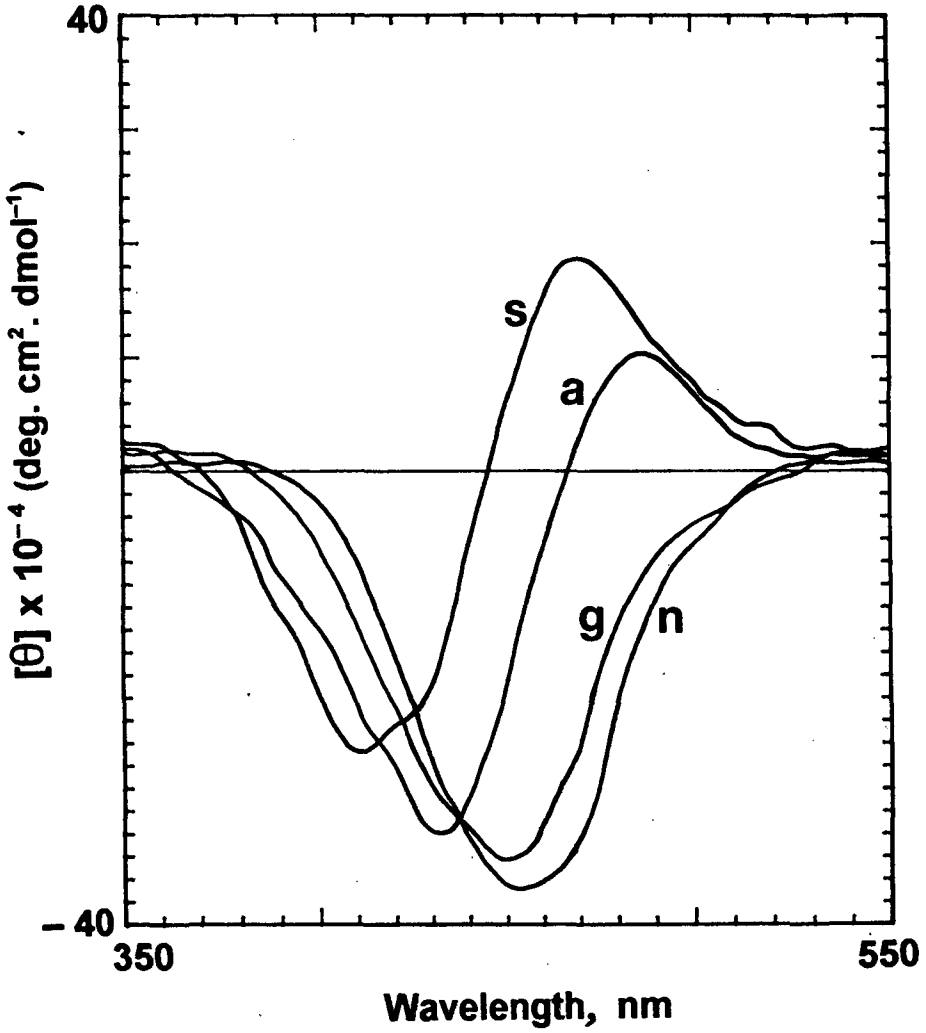


Figure 40. CD spectra of bilirubin (10μM) complexed with BuSA and its modified derivatives (20μM). Letters **n, g, a** and **s** stand for native, guanidinated, acetylated and succinylated BuSA derivatives respectively.

earlier observations (Lightner *et al.*, 1988) on the binding of various bilirubin isomers (having their carboxyl groups modified) to HSA. These isomers unlike parent bilirubin molecule generated weak CDCEs when bound to the same high affinity binding site on HSA indicating weak enantioselection in the absence of salt linkages. In view of this, our results also suggested the involvement of salt linkages existing between ε-NH_2 groups of internal lysine residues and carboxyl groups of bilirubin in the enantioselective incorporation of bilirubin into high affinity site on these serum albumins.

CD spectral results on the binding of bilirubin to covalent derivatives of GSA, SSA and BuSA were interesting. As can be seen from Figures 38, 39 and 40, characteristic monosignate CD spectra of bilirubin bound to native GSA, SSA or BuSA were transformed into bisignate CD spectra upon acetylation and succinylation (curves **a** and **s** respectively) of internal lysine residues in these albumins. The transformed CD spectra showed CD band minima (–) at shorter wavelength and maxima (+) at higher wavelength as compared to their native counterparts showing only monosignate negative CDCEs at higher wavelength (Table 5). The intensities of both positive and negative CDCEs of the transformed bisignate CD spectra were decreased significantly. These spectral characteristics were similar to the bisignate CD spectra of bilirubin-HSA or bilirubin-RbSA complexes (Figures 36 and 37, curve **n**). On the other hand, guanidination of internal lysine residues of these albumins (GSA, SSA and BuSA) did not change the shape and sign order of the

characteristic monosignate CD spectra of bound bilirubin but a shift in the CD band maxima and a slight decrease in the CDCE intensities were noticed (Figures 38, 39 and 40, curve g). It may be recalled that acetylation neutralizes the positive charge on ε-NH_2 groups of lysine residues whereas succinylation replaces the positive charge with a negative charge and in both cases, CD spectra of bound bilirubin were severely perturbed. On the other hand, guanidination of albumins where positive charge is retained on lysine residues, did not significantly alter the CD spectra of bilirubin-albumin complexes. From these results it appears that the presence of positive charge on internal lysine residue(s) plays an important role in the stereoselective binding of bilirubin involving salt linkage(s) between carboxyl groups of bilirubin and lysine residues of albumins and the absence of positive charge on these lysine residues (as found in acetylated and succinylated albumins) severely affects its chirality. The alteration in the chirality of the pigment bound to acetylated or succinylated albumin derivatives seems to be brought about by the change in the dihedral angle between the two chromophores of the bound pigment from the angle at which they are fixed when bilirubin is bound to native or guanidinated albumin derivatives.

Competition between native and modified albumins for bilirubin binding

Based on the bilirubin binding results described above, we showed the decrease in bilirubin binding affinity of different serum albumins upon acetylation and succinylation of internal lysine residues. To further assess the binding affinity, we

studied the switching of bound bilirubin from modified albumin derivatives to native albumins of the same species using CD spectroscopy. As can be seen from Figure 41A and B, bisignate CD spectra of the complexes of bilirubin with acetylated derivatives (curve 1) of both GSA and SSA were transformed to monosignate spectra (curve 2) after the addition of free GSA or SSA to these complexes. Monosignate CD spectra had the same characteristics as given by the complexes of bilirubin with native albumins. This indicated switching of bound bilirubin from acetylated GSA or SSA derivatives towards native albumins. Similar results were also obtained with succinylated derivatives of these albumins (figure omitted for clarity). No reversal in the switching of bound bilirubin from native GSA or SSA to acetylated or succinylated GSA or SSA derivatives was noticed upon addition of these derivatives to a solution containing bilirubin and native albumins (data not shown). These results suggested that acetylation and succinylation of internal lysine residues significantly altered the binding site geometry such that the ligand was not tightly bound and therefore switched rapidly to high affinity binding site available on native albumins.

Effect of chloroform on the CD spectra of bilirubin-albumin complexes

Involvement of lysine residue(s) in salt bridge(s) formation with carboxyl groups of bilirubin in bilirubin-albumin interaction was also studied by CD spectroscopy in the presence of chloroform as this anesthetic binds to the same high affinity bilirubin

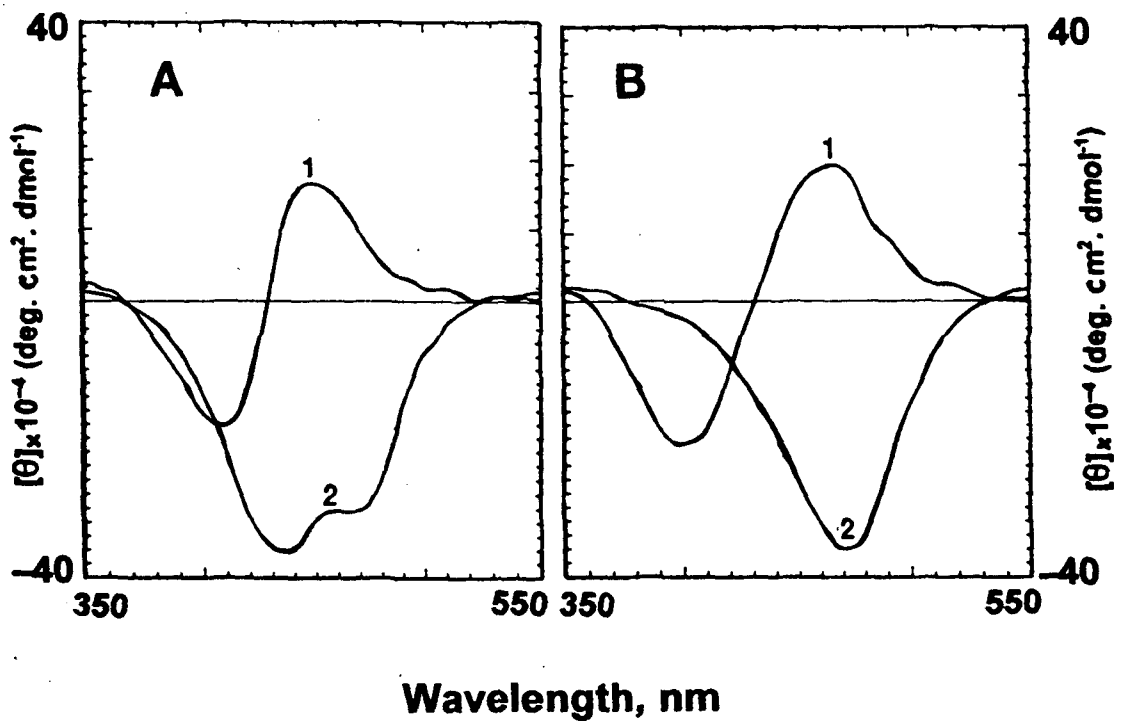


Figure 41. [A] Curve 1, CD spectrum of a bilirubin solution (10μM) containing acetylated GSA (20μM) recorded after 30-40min incubation of bilirubin-albumin solution in dark. Curve 2 represents CD spectra of the same solution after the addition of ~20μM of native GSA. [B] Curves 1 and 2 represent the CD spectral measurements involving acetylated and native SSA under similar experimental conditions as mentioned in the legend to Figure 41A.

binding site in albumins and alters the chirality of the bound pigment (McDonagh *et al.*, 1992; Pu *et al.*, 1993; Patra & Pal, 1997). The effect of chloroform on the CD spectra of bilirubin bound to different native albumins and their modified derivatives is shown in Figures 42-46. As can be seen from these figures, addition of a small amount of chloroform to the bilirubin-albumin solution, completely inverted the bisignate CD spectra of bilirubin complexed with HSA or RbSA (curve **n** of Figures 42 and 43 respectively). It also transformed the monosignate CD spectra of bilirubin complexed with GSA, SSA or BuSA into bisignate CD spectra (curve **n** of Figures 44, 45 and 46 respectively) similar to those obtained with a chloroform free solution of bilirubin-HSA and bilirubin-RbSA complexes (Figures 36 and 37 respectively, curve **n**). These observations were in agreement with previous reports suggesting that chloroform potentially alters the hydrophobic microenvironment of the binding site and thereby chirality of the bound pigment (McDonagh *et al.*, 1992; Pu *et al.*, 1993). As is clearly indicated from Figures 42, 44, 45 and 46 (curve **n**), there was an increase in the intensities of both positive and negative CDCEs of bilirubin complexed with HSA, GSA, SSA and BuSA, being much higher in case of bilirubin-SSA complex after chloroform addition as compared to the one obtained in the absence of chloroform. However, bilirubin-RbSA complex showed a decrease in the CDCEs' intensity (Figure 43, curve **n**) though the inversion of CD spectrum was similar to some extent to that of bilirubin-HSA complex. Increase in CDCEs' intensity upon chloroform addition to the complexes of bilirubin with guanidinated derivatives of these albumins was also

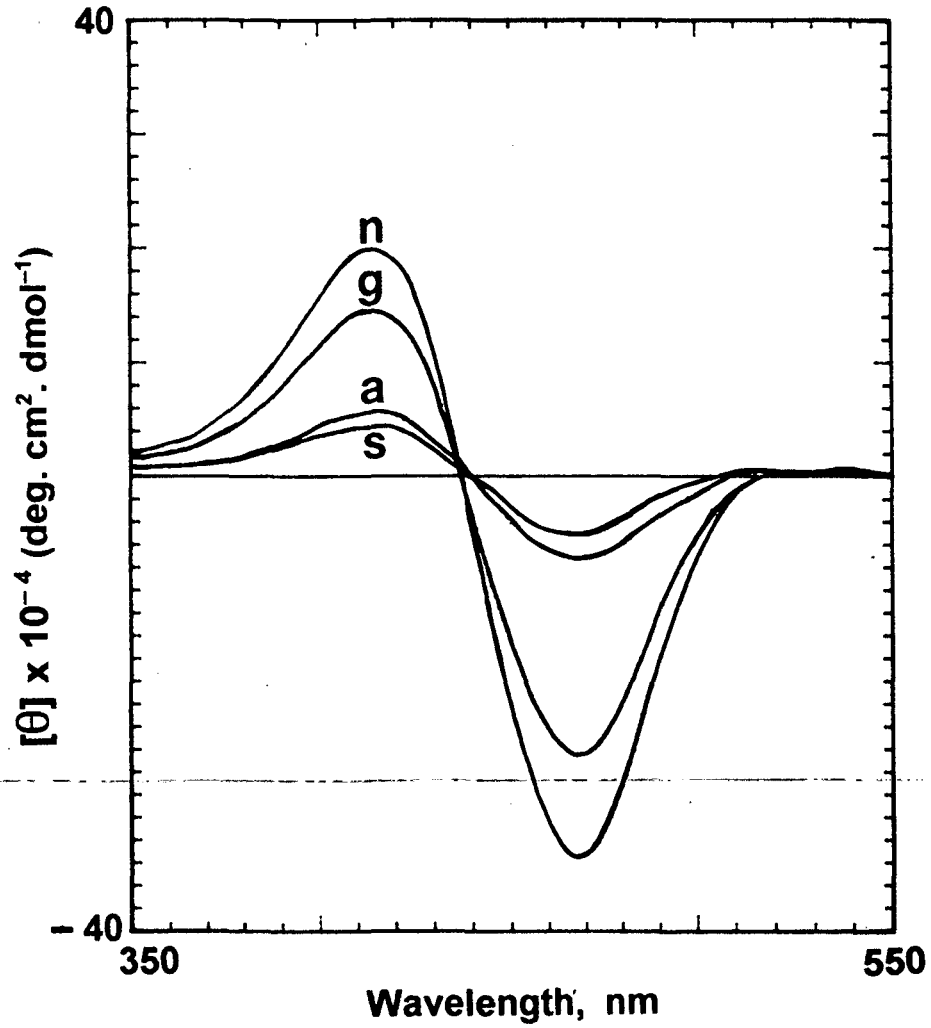


Figure 42. Effect of chloroform (~20mM) on the CD spectra of bilirubin (10μM) complexed with HSA and its modified derivatives (20μM). Letters **n, g, a** and **s** stand for native, guanidinated, acetylated and succinylated HSA derivatives respectively.

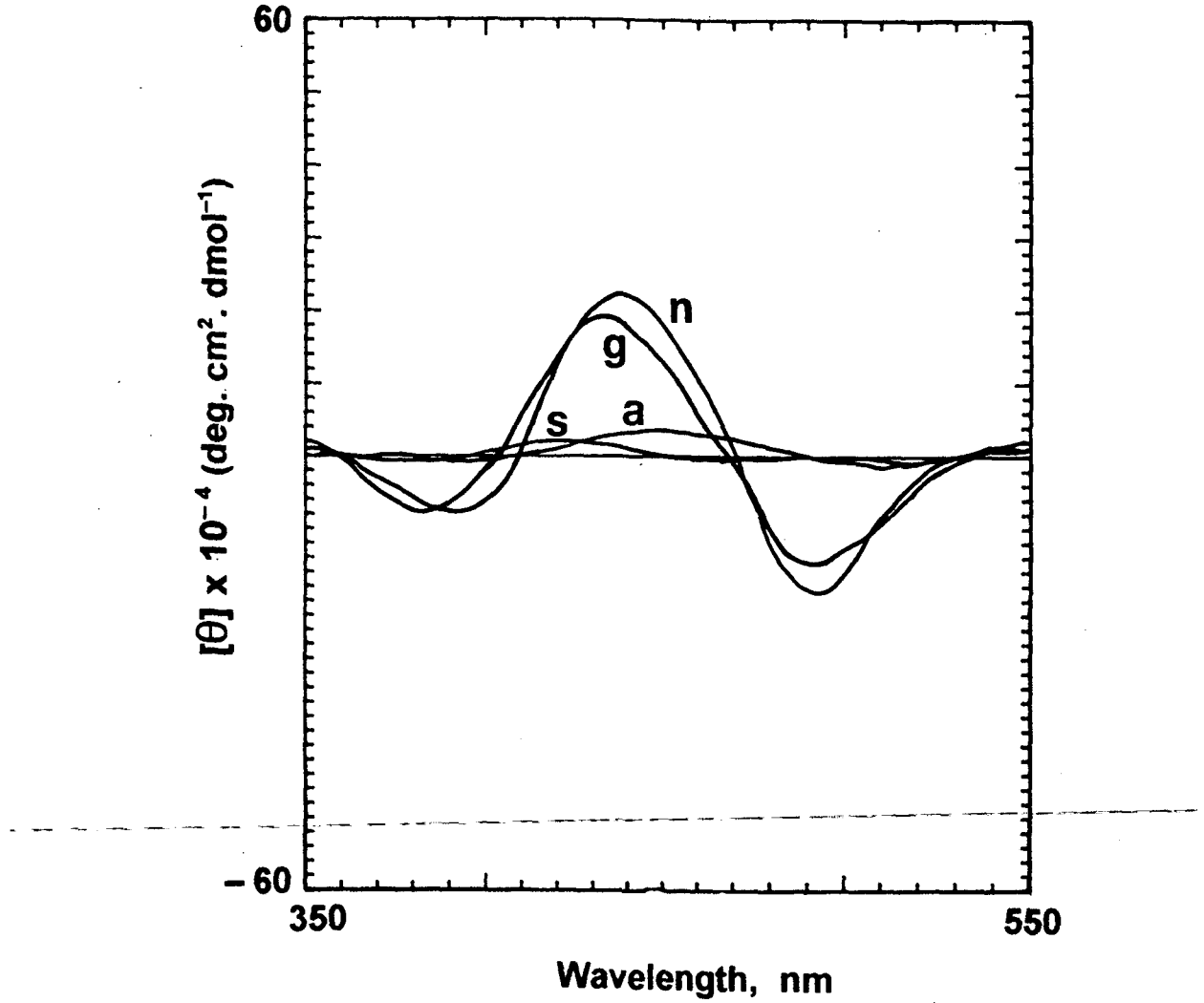


Figure 43. Effect of chloroform (~20mM) on the CD spectra of bilirubin (10μM) complexed with RbSA and its modified derivatives (20μM). Letters **n, g, a** and **s** stand for native, guanidinated, acetylated and succinylated RbSA derivatives respectively.

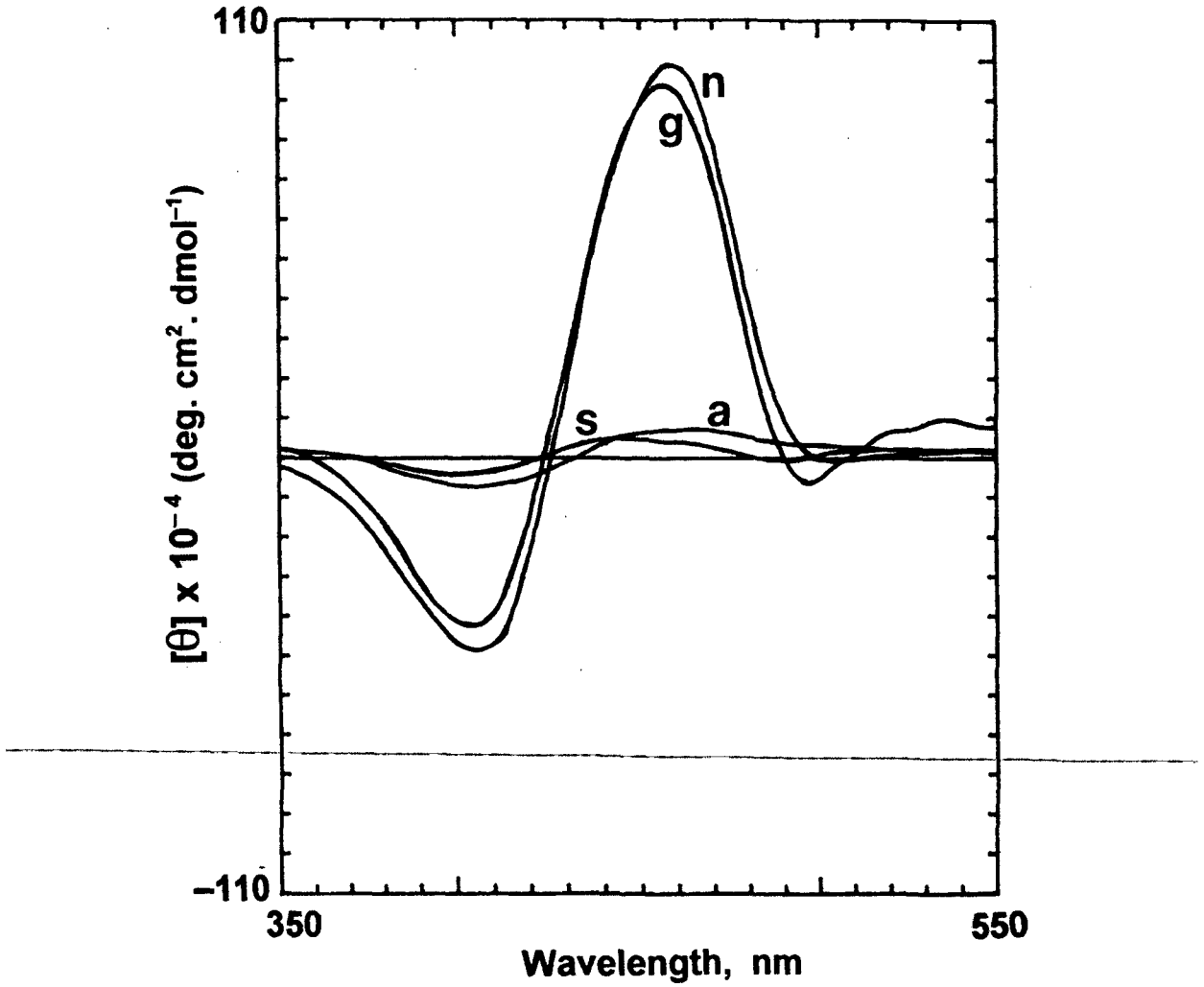


Figure 44. Effect of chloroform (~20mM) on the CD spectra of bilirubin (10μM) complexed with GSA and its modified derivatives (20μM). Letters **n, g, a** and **s** stand for native, guanidinated, acetylated and succinylated GSA derivatives respectively.

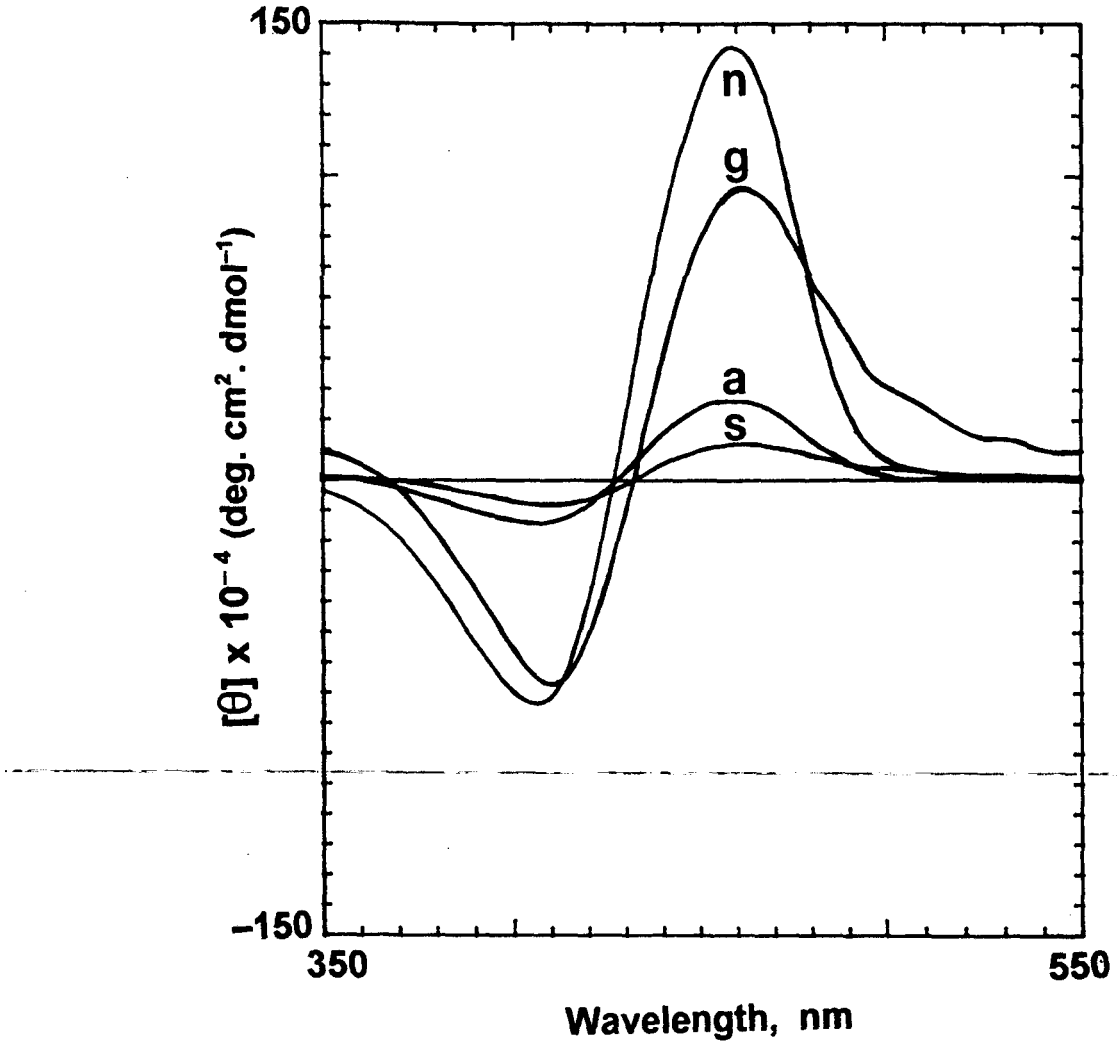


Figure 45. Effect of chloroform (~20mM) on the CD spectra of bilirubin (10μM) complexed with SSA and its modified derivatives (20μM). Letters **n, g, a** and **s** stand for native, guanidinated, acetylated and succinylated SSA derivatives respectively.

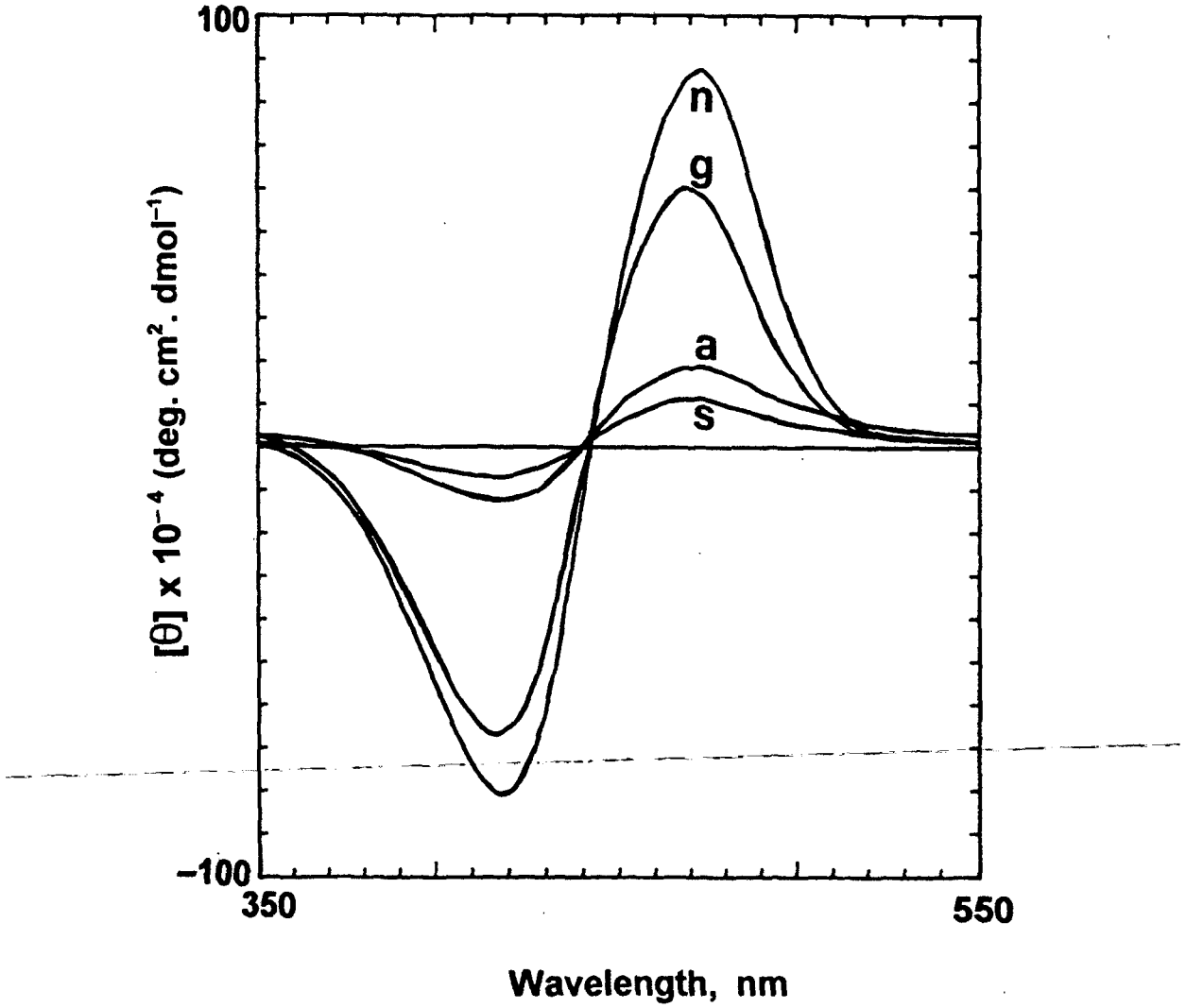


Figure 46. Effect of chloroform (~20mM) on the CD spectra of bilirubin (10μM) complexed with BuSA and its modified derivatives (20μM). Letters **n, g, a** and **s** stand for native, guanidinated, acetylated and succinylated BuSA derivatives respectively.

found to be similar to those obtained in case of bilirubin complexed with native albumins (Figures 42-46, curve g). The inversion and transformation of the CD spectra of different bilirubin-albumin complexes in the presence of chloroform can be attributed to the change in the stereochemistry of the bound pigment (Pu *et al.*, 1993). This change in the stereochemistry apparently favors the binding of bilirubin with a positive (*P*) chirality conformation (Lightner *et al.*, 1986; 1988) with enhanced enantioselectivity in the case of GSA, SSA and BuSA and with a negative (*M*) chirality conformation in HSA and RbSA (Pu *et al.*, 1993). Interestingly, the CD spectra of bilirubin complexed with acetylated or succinylated derivatives became insensitive to the presence of chloroform. Addition of chloroform to the complexes of bilirubin with acetylated and succinylated derivatives of these albumins (curves a and s respectively of Figures 42-46), drastically reduced the intensity of both positive and negative CDCEs being close to complete abolishment in case of GSA and RbSA. Further, except HSA (Figure 42), chloroform did not invert the bisignate CD spectra of bilirubin complexed with acetylated and succinylated derivatives of other albumins (curves a and s of Figures 43-46). These observations clearly suggest that the precise location and orientation of positively charged ε-NH_2 groups of lysine residue(s) at the ligand binding site and their involvement in salt bridge(s) formation with carboxyl group(s) of bilirubin are crucial for efficient and effective stereochemical changes in the bound pigment brought about by co-binding of chloroform (McDonagh *et al.*, 1992; Pu *et al.*, 1993; Patra & Pal, 1997).

From the above results it can be said that one or two of the internal lysine residue(s) of these serum albumins play an important role in bilirubin-albumin interaction through salt bridge(s) formation with oppositely charged carboxyl groups of bilirubin. The replacement of positive charge on these lysine residues severely perturbed the enantiomeric stereoselectivity of ligand at high affinity site on these albumins.

Photochemical changes of bilirubin bound to HSA and its derivatives

When an equimolar (1:1) complex of bilirubin with HSA was irradiated under white light for different time periods (0-30 minutes), the absorbance decreased continuously with a slight shift (5 nm) in the absorption maxima (Figure 47). Guanidinated HSA showed a similar pattern to that observed with native HSA, however, the blue shift was more significant (11 nm) after irradiation for 30 minutes. On the other hand, under similar conditions, a brief irradiation (2 minutes) of the complex of bilirubin with either aHSA or sHSA produced a significant rapid shift of 15 nm in the absorption maxima towards a shorter wavelength which continued up to 4-5 minutes and then became constant at 435 nm and 434 nm in aHSA and sHSA, respectively (Figure 47). A decrease in absorbance as well as the blue shift observed in the absorption spectrum of the bilirubin-HSA complex upon irradiation can be ascribed to the photoconversion of bilirubin into photobilirubins. A marked blue shift observed in the bilirubin-aHSA and bilirubin-sHSA complexes after 4 minutes irradiation seems to be due to the fast conversion of bilirubin into cyclobilirubin which absorbs at 435 nm (Migliorini *et al.*,

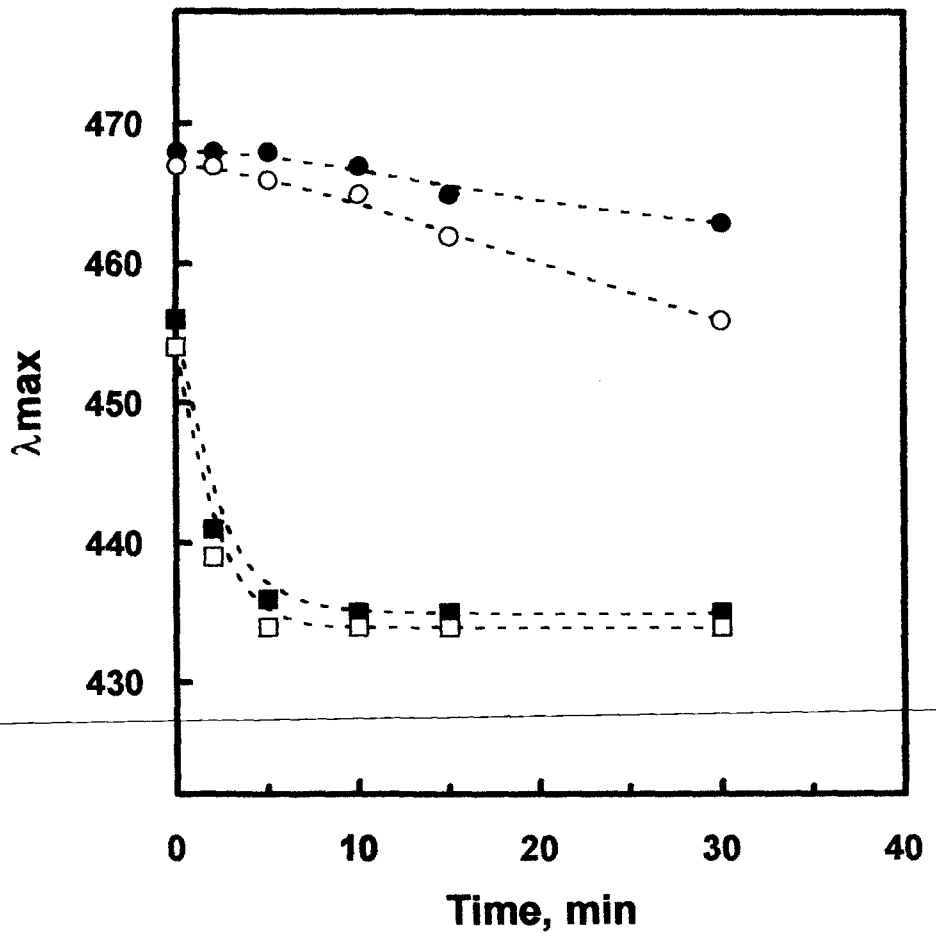


Figure 47. Effect of time on the absorption maxima (λ_{max}) of bilirubin complexed with HSA (•) and its modified derivatives viz. gHSA (◦), aHSA (■) and sHSA (□) upon irradiation with white light.

1988). In view of the involvement of electrostatic interactions between a negatively charged bilirubin dianion and positively charged lysine residues of albumin in the bilirubin-albumin interaction, it seems reasonable that this salt linkage provides some stability to the bilirubin chromophore which is lost when it is broken as found in the case of aHSA and sHSA, respectively. These results indicated that a brief irradiation of the complex of bilirubin with either aHSA or sHSA weakened this interaction and bilirubin bound to these derivatives then followed a photochemical path similar to that of free bilirubin. However, no significant shift (Figure 47) in the absorption maxima was noticed, even up to 15 minutes irradiation of an equimolar complex of bilirubin with HSA, though the absorbance decreased continuously. A similar pattern was shown by gHSA (Figure 47) in which a small shift was observed upon 30 minutes irradiation. These results suggest the role of salt bridges provided by ε-NH_2 groups of lysine residues of HSA and bilirubin dianion in the kinetics of photoisomerization of the bound pigment. Taken together, the results obtained in earlier studies (Onishi *et al.*, 1980; 1985; Lamola *et al.*, 1981; McDonagh *et al.*, 1982; 1989; Costarino *et al.*, 1985) and our results on photochemical changes of bilirubin bound to aHSA and sHSA, it appears likely that ε-NH_2 groups of lysine residues in the primary bilirubin binding site of HSA, are directly involved in stereospecific and regioselective photoisomerization of bound bilirubin.

Figure 48A shows the effect of visible light on the induced fluorescence of bilirubin bound to HSA and its derivatives. As can be seen from the figure, continuous

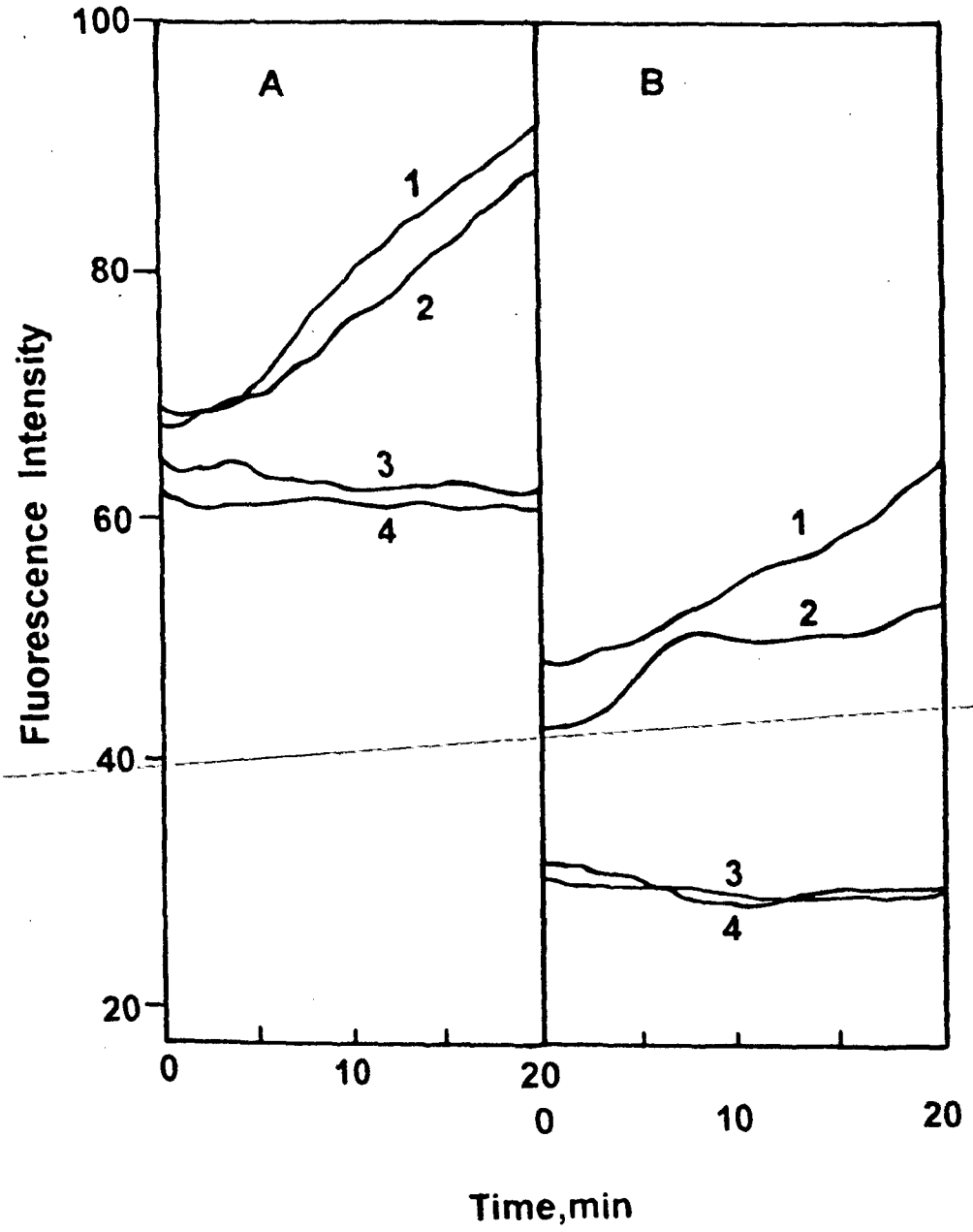


Figure 48. [A] change in fluorescence intensity of bilirubin-albumin complexes (20μM albumin and 10μM bilirubin) at 530 nm as a function of dose of irradiation at 468nm. Curves 1, 2, 3 and 4 represent the fluorescence spectra of bilirubin-HSA, bilirubin-gHSA, bilirubin-aHSA and bilirubin-sHSA complexes respectively. [B] fluorescence intensity change was recorded under identical conditions as in Figure A but the solutions contained ~20mM chloroform in each sample.

irradiation of the samples in spectrofluorometer at 468 nm up to 20 minutes led to a small decrease or no change for 2 minutes followed by a continuous increase in the fluorescence intensity in both bilirubin-HSA (curve 1) and bilirubin-gHSA (curve 2) complexes. Astonishingly, when bilirubin-aHSA or bilirubin-sHSA complexes (curves 3 and 4 of Figure 48A) were irradiated under similar experimental conditions, there was no significant change in the fluorescence intensity. Further, increasing the time of irradiation up to 1 hour did not affect the fluorescence intensity of these complexes. Similar pattern of the change in fluorescence intensity was observed when the samples were irradiated at 440 nm (data not shown). Since bilirubin bound to HSA shows maximum absorbance at 468 nm whereas unbound bilirubin absorbs maximally at 440 nm, this wavelength was selected to irradiate the samples of bound bilirubin to preclude the involvement of free bilirubin in these changes. It is of importance to note that neither free (unbound) bilirubin fluoresces (Chen, 1973; 1974) nor the fluorescence is induced when continuously irradiated at these wavelengths. The fluorescence enhancement seen in bilirubin-HSA complex upon photoirradiation was in good agreement with earlier findings (Lamola *et al.*, 1981; 1982). Complex of bilirubin with gHSA derivative (in which positive charge was retained on critical lysine residues) showed similar behavior upon photoirradiation. On the other hand, photoinduced fluorescence enhancement of bound bilirubin was completely ceased in both acetylated and succinylated HSA derivatives in which positive charge on critical lysine residues was abolished and replaced by zero charge

and a negative charge respectively. Here, it is important to note that bilirubin bound to these derivatives (aHSA and sHSA) showed bisignate CD spectrum similar to the one obtained with native HSA (Figure 36). However, concomitant modulations in the bisignate CD spectrum of bilirubin complexed to these derivatives were noticed upon photoirradiation (Figure 49A and B). These results reflected the importance of positive charge on ε-NH_2 groups of lysine residues of the protein, involved in salt linkage(s) formation with the carboxyl groups of bilirubin as predicted earlier (Lightner *et al.*, 1986; 1988) as their masking can markedly alter the dynamics and stereoselectivity of photoinduced asymmetric transformation of the bound pigment.

Several lines of evidence suggest that high affinity bilirubin binding site on HSA is located in the protein interior corresponding to a hydrophobic pocket in subdomain IIA (Carter & Ho, 1994). This region is highly stable and is generally not destabilized by urea and even at extremely low pH, this region is characterized by abundant secondary structure and few native like tertiary folds (Muzammil *et al.*, 1999; 2000; Dockal *et al.*, 2000). Therefore, one may argue that covalent masking of critical lysine residues located in this region by bulky and sterically unfavorable *acetyl*, *succinyl* and *guanidino* groups may induce structural alterations in the protein which may decrease the affinity of bilirubin for the primary binding site and therefore bilirubin probably sinks into secondary low affinity sites where its photoinduced spectral properties are not comparable to those observed when bound to primary high affinity site. This seems unlikely for two reasons: first as characterized by far-UV

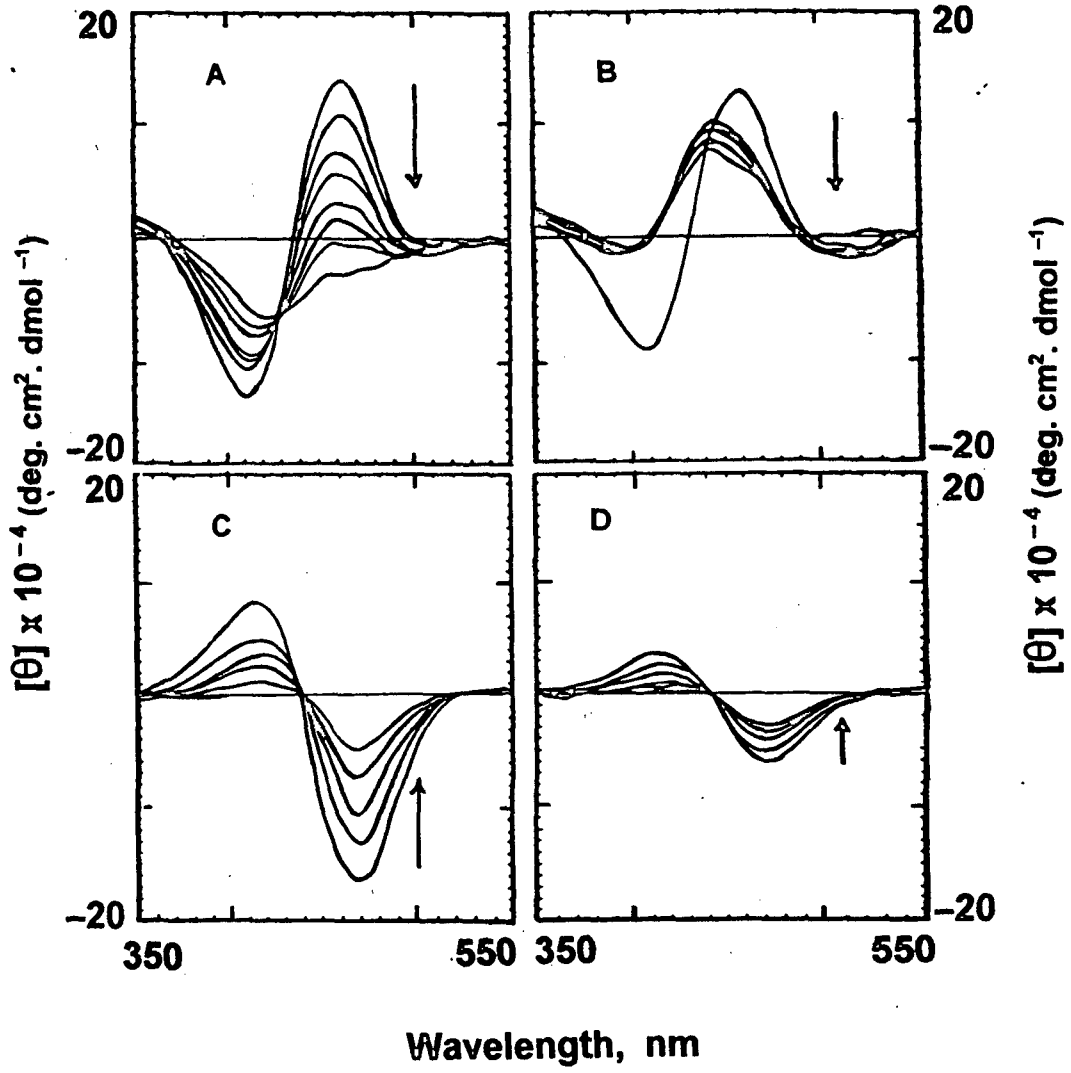


Figure 49. CD spectral changes accompanied by irradiation of bilirubin complexed with, [A] HSA (10μM albumin and 5μM bilirubin) and [B] aHSA (11.5μM albumin and 6μM bilirubin). Dose of irradiation from top to bottom was: 0, 1, 2, 3, 6, 10, 15 and 30 min. [C] and [D] show the effect of chloroform (~20mM) on the CD spectral changes of bilirubin-HSA and bilirubin-aHSA complexes respectively. Dose of irradiation from bottom to top was: 0, 2, 6, 15 and 30min.

CD measurements, no detectable secondary structural changes were induced in HSA by covalent modification though near-UV CD spectra of aHSA and sHSA were only slightly perturbed (Figure 20A and B respectively) and second that photoinduced spectral modulations were not due to the binding of bilirubin to secondary low affinity site(s) because with all the HSA derivatives, CD spectrum of bound bilirubin was completely inverted upon addition of chloroform (Figure 42) which evidently binds to subdomain IIA in HSA (Eckenhoff, 1996; Johansson, 1997) where primary bilirubin binding site is located (Carter & Ho, 1994). Presence of chloroform caused a red shift of 3-13 nm in the position of inverted CD bands and decreased the intensity of both positive and negative CDCEs in case of bilirubin-aHSA and bilirubin-sHSA complexes (Figure 42). The decrease in CDCEs' intensity can not be ascribed to weaker chloroform binding to these derivatives (aHSA and sHSA) as similar quenching of tryptophan fluorescence was observed in these derivatives in the presence of chloroform (Figure 50). Chloroform at higher concentration (26mM) also produced a significant blue shift (~ 6-10 nm) in the tryptophan emission maxima of HSA (Figure 50) and its derivatives (figure omitted for brevity) which was indicative of the changes in the dielectric microenvironment (towards more apolar region) of indole ring in tryptophan (Johansson, 1997). These results suggest that inversion in the pigment chirality (as reflected by inverted CDCEs) and red shift in the CD band maxima are due to alterations in the hydrophobic microenvironment and internal topography of the primary pigment binding site induced by chloroform

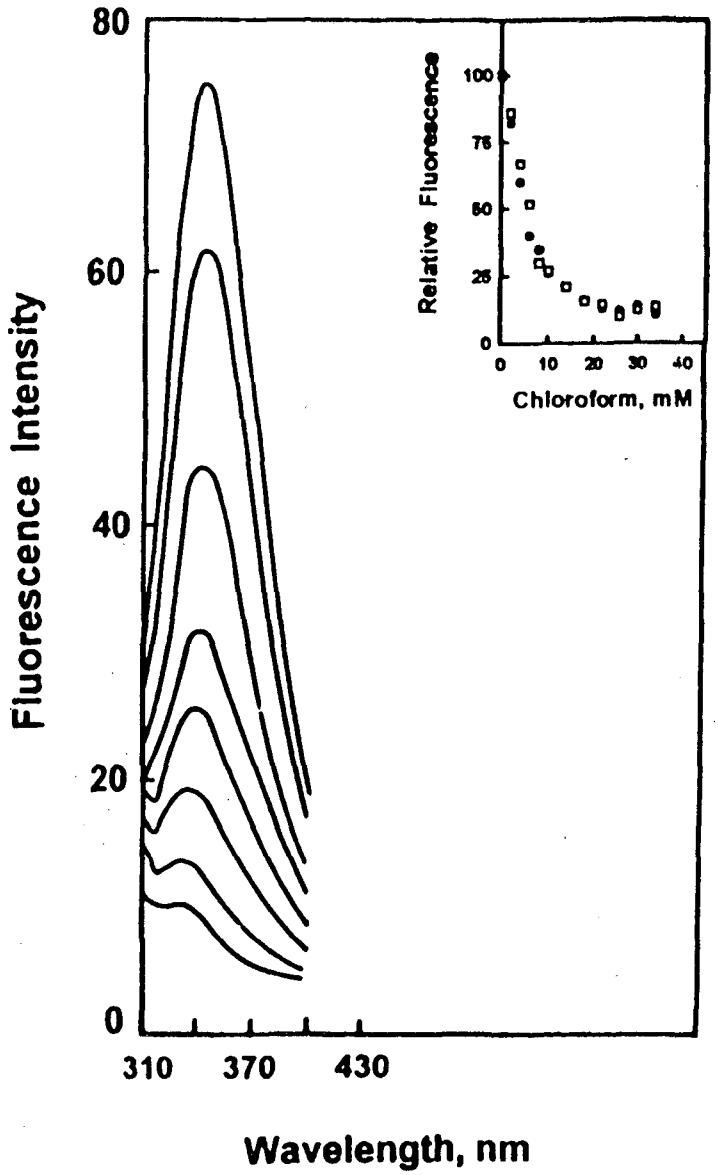


Figure 50. Effect of chloroform on tryptophan fluorescence of HSA (5μM) at pH 7.4. Chloroform concentration from top to bottom was varied as 0, 2, 4, 6, 8, 10, 18 and 26mM. Inset shows the tryptophan fluorescence quenching of (•) HSA and (□) aHSA at increasing chloroform concentration. Each data point was the average of three independent determinations. The protein was excited at 295nm and emission was recorded at 342nm.

(McDonagh *et al.*, 1992; Pu *et al.*, 1993). Addition of chloroform to the bilirubin-albumin complexes also decreased the fluorescence yield of bound bilirubin (Figure 48B), being 50% of that seen in the absence of chloroform in case of bilirubin-aHSA and bilirubin-sHSA complexes (Figure 48B, curves 3 and 4). This indicates that bilirubin is still binding in IIA subdomain of modified albumin and the decrease in fluorescence is due to chloroform binding in the close proximity of bilirubin (Johansson, 1997). From these results it appeared that it was not the specificity rather affinity of bilirubin for the primary binding site on HSA which was decreased by covalent modification. Since chloroform binds to different HSA derivatives with more or less equal affinity as that for native HSA as evident from the quenching results (Figure 50), it can be said that covalent blocking of ε-NH_2 groups particularly those located in the protein interior does not alter the anesthetic binding site which is hydrophobic in nature. Therefore, electrostatic rather than hydrophobic interactions seem to play an important role in the photoinduced spectral modulations of bound bilirubin in bilirubin-albumin complexes.

The CD spectra of bilirubin complexed with different HSA derivatives (gHSA, aHSA and sHSA) were characterized by bisignate CDCEs (Figure 36, curves 2, 3 and 4, respectively) similar to that bound to native HSA (Figure 36, curve 1). A brief (1 minute) irradiation of bilirubin bound to native HSA under white fluorescent light, produced only little change in the magnitude of both positive and negative CDCEs (Figure 49A). Similar changes were also observed in case of

bilirubin-gHSA complex (data not shown). On the other hand, drastic changes were observed in case of bilirubin bound to aHSA and sHSA in which a brief irradiation of the bound bilirubin under white fluorescent light, completely abolished the negative CDCEs while the intensity of positive CDCEs was moderately decreased and the band maxima shifted towards blue region (Figure 49B). As can be seen from Figure 49A and B, a 15 minutes dose of irradiation produced different results with bilirubin-HSA and bilirubin-aHSA complexes such that the spectra were characterized by monosignate CDCE of opposite sign which indicated that upon irradiation to white light, the bilirubin molecule in the binding pocket of HSA acquired different conformational state in the absence of salt bridge(s) (as found in aHSA and sHSA) compared to the one in which salt bridges were present (as found in HSA and gHSA). Presence of chloroform reconciled CD spectral modulations of bilirubin-aHSA and bilirubin-sHSA complexes as similar photoinduced changes were noticed in the CD spectrum of bilirubin bound either to native HSA or its derivatives (Figure 49C and D). In view of the stabilizing effect of chloroform on certain folded conformation of proteins (Tanner *et al.*,1999; Johansson *et al.*, 1999), it can be said that stabilization of certain local regions at bilirubin binding site in aHSA and sHSA by chloroform, provides stability to the bound pigment against light as evident from identical CD spectral changes upon photoirradiation (Figure 49C and D). These observations suggest that anesthetics not only alter the local protein dynamics (Johansson *et al.*, 1999) but also attenuate the dynamics or mobility of those

nearby bound ligands whose conformation is sensitive (e.g., bilirubin) to the presence of anesthetics (Pu *et al.*, 1993). Interestingly, chloroform-induced enantiomeric stabilization did not bring fluorescence of bilirubin-aHSA or bilirubin-sHSA complexes (Figure 48B, curves 3 and 4) close to that produced by bilirubin-HSA or bilirubin-gHSA complexes upon photoirradiation (Figure 48B, curves 1 and 2).

Bilirubin, a bichromophoric tetrapyrrole, is characterized by an extensive conjugated double bond system. Although unbound pigment absorbs strongly in the visible region, it does not fluoresce. The fluorescence is induced when it forms complexes with chiral binding agents like albumin (Chen, 1973; 1974). It has been suggested that the genesis of fluorescence in bound bilirubin is due to acquiring helical shape rather than hydrophobic stacking (Chen, 1973; 1974). Various bilirubin isomers also acquire a helical shape as revealed by induced fluorescence and circular dichroism (Lamola *et al.*, 1983; Lightner *et al.*, 1986; 1988). Thus, once the fluorescence is induced upon binding to HSA due to helix formation, then what accounts for its further enhancement upon exposing the bound pigment to visible light? Conformational twisting of bound bilirubin leading to configurational or structural isomerization (endo- vinyl cyclization) upon photoirradiation is well known (Lamola *et al.*, 1981; 1982; 1983; McDonagh & Lightner, 1985). Whether these processes are freely taking place or under some restrictions, and if restricted then by which force, is not very clear. From the results on the further enhancement of

fluorescence (Figure 48A, curves 1 and 2) and slow and steady decrease in positive CDCEs (Figure 49A) upon photoirradiation of bilirubin-HSA and bilirubin-gHSA complexes and the absence of these phenomena in both bilirubin-aHSA and bilirubin-sHSA complexes (Figure 48A, curves 3 and 4 and Figure 49B), it seems that conformational twisting of bound bilirubin under some restrictions, posed by salt linkage(s) between ε-NH_2 groups of HSA and carboxyl groups of bilirubin, is responsible for photoinduced fluorescence enhancement. It has been reported earlier that photoirradiation of bilirubin bound to albumin or free bilirubin dissolved in organic solvent (chloroform) results in the formation of the same photoisomerized species (Lamola *et al.*, 1981). Thus, if photoisomerization is supposed to be the cause of fluorescence enhancement, then bilirubin dissolved in chloroform should also exhibit fluorescence upon photoirradiation. However, no fluorescence was induced in free bilirubin dissolved in chloroform upon photoirradiation, an observation which has been also been reported by earlier workers (Lamola *et al.*, 1981; 1982; 1983). Interestingly, bilirubin bound to aHSA and sHSA though showed induced fluorescence but failed to produce any further enhancement in fluorescence upon photoirradiation. Therefore, it is not merely the configurational or structural photoisomerization accompanied by delocalization of π-electrons of the pyrrole rings as well as vinyl groups of bound bilirubin responsible for further enhancement of fluorescence. It appears that along with all these processes, the whole pigment is sterically strained due to salt linkage(s) formation involving its carboxyl groups

and ε-NH_2 groups of protein and the two locally excited pyrromethanone chromophores (excitons) of the bound pigment held in a folded conformation (Lightner *et al.*, 1986; 1988; Person *et al.*, 1994), interacting strongly (exciton coupling) with each other by resonance splitting or the bound pigment undergoes tight helical twisting as the photoirradiation proceeds, causing the fluorescence to increase further (Figure 48A, curve 1). It has been reported that absorption of light by bilirubin leads to a faster photochemical reaction involving rotation of one of the end ring at 180 degree about the double bond followed by a slow reaction leading to cyclization of the pigment (Lamola *et al.*, 1983; McDonagh & Lightner, 1985). Photoirradiation is indeed accompanied by an initial small decrease in the fluorescence intensity of the bound pigment followed by a slower increase several times higher than the initial fluorescence intensity, as shown earlier (Lamola *et al.*, 1981; 1982) and also observed in this study (Figure 48A). The slower increase in fluorescence was completely ceased in case of bilirubin-aHSA or bilirubin-sHSA complexes (Figure 48A, curves 3 and 4). These observations indicated that although bilirubin bound to aHSA or sHSA acquired similar folded conformation (helical) as observed with native HSA based on similarity in the bisignate CD spectrum as well as induced fluorescence upon binding to these derivatives (Figures 36 and 48A), this helicity did not in any way allow the fluorescence of bound bilirubin to increase further upon photoirradiation. It means that masking of critical ε-NH_2 groups in HSA thereby abolishing salt linkage(s) formation between bilirubin and HSA

(Lightner *et al.*, 1988; Person *et al.*, 1994) does not interfere with the binding of bilirubin in a folded ridge-tile conformation which is in corroboration with earlier findings on the binding of various bilirubin isomers to HSA (Lightner *et al.*, 1986; 1988). However, the instability of the bound pigment in bilirubin-aHSA and bilirubin-sHSA complexes was clearly reflected from a very rapid elimination of negative CDCEs upon photoirradiation for a brief time period. This seems to be possible as in the absence of salt linkage(s) between pigment and protein in these complexes, either one or both of the pyrromethanone units of the bound bilirubin is (are) supposed to have more freedom to rotate at $-CH^{10}-$ along with the rotation of one of the end pyrrole ring but this rotation is certainly different from that allowed when bound to native HSA. In this rotation, the two locally excited pyrromethanone chromophores either do not interact or interact very weakly, thereby causing alteration in the helicity due to which the fluorescence of bound bilirubin does not increase upon photoirradiation. This is understandable because when bilirubin complexed with aHSA or sHSA was photoirradiated briefly (1 minute), its bisignate CDCEs were rapidly transformed into monosignate CDCE of positive sign (Figure 49B) akin to that exhibited by xanthobilirubin bound to HSA (Lamola *et al.*, 1983; Lightner *et al.*, 1986). This suggested that a brief photoirradiation of these complexes left only one half of bilirubin in contact with the binding site elements and the other half free in such a way that the phenomenon of exciton coupling / exciton interaction between the two excited pyrromethanone chromophores (Lightner *et al.*, 1986; 1988;

Person *et al.*, 1994) of the bound pigment did not occur and therefore, its fluorescence did not increase upon photoirradiation. This is exactly similar to xanthobilirubin bound to HSA showing induced fluorescence which does not increase further upon photoirradiation (Lamola *et al.*, 1983).

Various binding forces have been suggested to facilitate the proper binding of bilirubin to HSA (Brodersen, 1979; 1982). Among these, chiral amine salt linkage(s) and associated intermolecular hydrogen bonds are considered to be the dominant forces involved in the enantioselective incorporation of the pigment at high affinity site (Brodersen, 1979; 1982; Lightner *et al.*, 1986; 1988; Person *et al.*, 1994). Results presented above revealed another important implication of salt linkage(s) present in bilirubin-HSA complex in the dynamics and stereoselectivity of photoinduced conformational changes in the bound pigment. Thus, the precise location of ε-NH_2 groups at the primary binding site on HSA is only one requirement for the binding orientation and enantioselectivity of the pigment (Lightner *et al.*, 1986; 1988); the other includes restriction of the conformational rotation of the bound pigment accessible by photoactivation. Since photoinduced excitation of π-electrons may lead to partial or complete disruption of aromatic π–π van der Waals attractions existing between pyrromethanone chromophores of bilirubin and tryptophan and tyrosine residues of protein and therefore along with these van der Waals attractions, the secondary hydrogen bondings, i.e., not associated with salt linkages (Brodersen, 1979; 1982; McDonagh & Lightner, 1985; Lightner *et al.*, 1986;

1988; Person *et al.*, 1994); presumably seem insufficient to stabilize the bound pigment against light in the absence of salt linkage(s).

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On the modulation of photoinduced fluorescence enhancement and conformational stability of albumin-bound bilirubin: Effect of ε-NH_2 groups blocking and chloroform binding

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Abstract

Photoinduced fluorescence enhancement of bilirubin bound to primary binding site on human serum albumin (HSA) was completely ceased when ε-NH_2 groups of its internal lysine residues were covalently blocked by acetylation or succinylation though the pigment bound to these derivatives in a folded conformation akin to that bound to HSA. These photoinduced fluorescence modulations cannot be ascribed to the binding of bilirubin to secondary low affinity sites as the CD spectrum of bilirubin bound to these derivatives showed complete inversion upon addition of chloroform which binds to subdomain IIA in HSA where high affinity bilirubin binding site is located. Presence of chloroform reconciled the photoinduced alterations in the CD spectrum observed in its absence, suggesting that chloroform stabilized the bound ligand against light but the fluorescence properties of bilirubin complexed with acetylated or succinylated derivatives remained unchanged. Guanidination of internal ε-NH_2 groups in HSA by *O*-methylisourea did not alter the spectral properties of the bound ligand. These results suggest that salt linkage(s) existing between ε-NH_2 groups of lysine residues in HSA and carboxyl groups of bilirubin, act(s) as a potential barrier during conformational rotation of the bound ligand assisted by photoactivation and their abolishment can alter its dynamics and stereoselectivity, a hitherto unnoticed implication of salt linkage(s) in BR–HSA complex.

Keywords: Albumin-bound bilirubin; Chemical modification; Chloroform-induced stabilization; Conformational dynamics; Fluorescence modulation; Photoirradiation; Salt linkage

1. Introduction

Bilirubin (BR), the principal degradation product of heme metabolism in mammals is transported to the liver by albumin for conjugation and subsequent excretion from the body [1–3]. The binding of BR to human serum albumin (HSA) has been a long term discussion with reference to the, binding forces involved in BR–HSA interaction, nature of the binding site and conformation of the bound pigment [4–7]. As revealed by circular dichroism (CD) spectroscopy that BR binds to HSA in a dissymmetric folded ridge-tile conformation [6,7]. In this conformation, the bound pigment shows bisignate CD Cotton effects (CDCEs), a characteristic of intramolecular exciton coupling between two pyrromethanone chromophores and exhibits greatly increased fluorescence [6,8]. Though BR consists of extensive conjugated double bond system but it is the helix formation in the pigment upon binding to albumin that has been suggested to be the cause of induced fluorescence [8,9]. The fluorescence of albumin bound BR further increased when irradiated under visible light whereas intensity of CDCEs of the bound pigment decreased significantly [10,11]. Recently we have reported that covalent modification of ε-NH_2 groups of critical lysine residues in HSA (involved in salt linkage(s) formation with carboxyl groups of BR) by acetylation or succinylation changed the pattern of photoinduced alterations in the CD as well as fluorescence spectra of the bound pigment [12]. The fluorescence of BR bound to acetylated or succinylated HSA derivatives did not increase when irradiated under white fluorescent light for varying times though the pigment bound to these derivatives showed bisignate CDCEs suggesting the binding of a folded ridge-tile conformer with positive (*P*) chirality akin to that bound to native HSA [6,7]. There is uncertainty that whether BR binds to the same high affinity site on

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Role of salt bridge(s) in the binding and photoconversion of bilirubin bound to high affinity site on human serum albumin

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Abstract

The role of salt bridge(s) (between ε-NH_2 groups of lysine residues of human serum albumin (HSA) and carboxyl groups of bilirubin) in the binding and photoconversion of bilirubin bound to high affinity site on HSA was investigated by covalent modification of ~20% internal (buried) lysine residues of HSA with acetic anhydride, succinic anhydride and *O*-methylisourea and white light irradiation of their complexes with bilirubin. The different HSA derivatives, namely, acetylated HSA (aHSA), succinylated HSA (sHSA) and guanidinated HSA (gHSA), thus obtained, were found to be homogeneous with respect to charge and size and characterized in detail in terms of mean residue ellipticity, Stokes radius, tryptophan fluorescence, bilirubin binding and the photochemistry of their complexes with bilirubin. All the three derivatives retained helical contents and molecular size (Stokes radius) similar to HSA except for sHSA which showed a slight increase in the Stokes radius from 3.56 to 3.64 nm. Further, fluorescence properties of aHSA and sHSA were also found to be different from HSA and gHSA. Based on difference spectral change, fluorescence quenching and fluorescence enhancement results of bilirubin bound to HSA and its derivatives, nearly 46 and 48% reduction in bilirubin binding was observed in the case of aHSA and sHSA, respectively. Both aHSA and sHSA showed a decrease of 8- and 10-fold, respectively, in association constant compared to native HSA. Although the bisignate circular dichroism (CD) spectra of an equimolar (1:1) bilirubin–HSA complex was retained by all three HSA derivatives, the intensity of both positive and negative CD Cotton effects decreased significantly in both aHSA and sHSA. gHSA which retained net charge identical to native HSA, showed little decrease in bilirubin binding and the intensity of bisignate CD Cotton effects. The photochemical reaction of bilirubin bound to aHSA and sHSA produced opposite results to those observed with HSA and gHSA. A brief (2 min) irradiation of an equimolar complex of bilirubin with both aHSA and sHSA accompanied a rapid shift (14–15 nm) in the absorption spectrum of the bound pigment towards the blue region and almost complete elimination of negative CD Cotton effects while only moderately affecting the magnitude of positive CD Cotton effects. On the other hand, similar treatment of the complexes of bilirubin with HSA and gHSA did not show any change in the absorption spectrum, only a slight decrease in the intensity of both positive and negative CD Cotton effects was observed. The fluorescence intensity of bilirubin bound to HSA and gHSA was increased upon irradiation with white light and after 30 min it was nearly twice the value observed at 0 min irradiation. Interestingly, no change in the fluorescence intensity of bilirubin bound either to aHSA or sHSA was observed upon irradiation, even on increasing the duration of irradiation to 1 h. Taken together, the results on fluorescence quenching, fluorescence enhancement, CD spectral changes and visible absorption spectroscopy suggest that salt bridge(s) of the type

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Chloroform-induced conformational changes in the bound pigment in bilirubin-albumin complexes

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)stract — Chloroform-induced conformational changes of bilirubin (BR) bound to different serum albumins were studied by ·cular dichroism (CD) and fluorescence spectroscopy. Addition of a small amount of chloroform (≈ 20 mM) to a solution containing) μM albumin and 15 μM BR changed the sign order and magnitude of the characteristic CD spectra of all BR-albumin complexes cept BR-PSA complex which showed abnormal behavior. Monosignate negative CD Cotton effects (CDCEs) of BR complexed with ;A. GSA and BuSA were transformed into bisignate CDCEs in presence of chloroform akin to those exhibited by chloroform free lution of BR-HSA complex, indicating that the pigment acquired right handed plus (P) chirality when chloroform was added to these mplexes. Bisignate CD spectra of BR complexed with HSA and BSA showed complete inversion upon addition of chloroform rroborating earlier findings. On the other hand, changes observed with BR-RSA complex were slightly different showing an ıditional CD band of weak intensity centered around 390 nm though inversion of CDCEs was similar to that of BR-HSA complex. onosignate CD spectra of BR-PSA complex also showed three CD bands occurring at 409, 470 and 514 nm after chloroform ldition. These results indicated significant but different effects of chloroform on the conformation of bound BR in BR-albumin mplexes which can be ascribed to the changes in the exciton chirality of bilirubin probably due to altered hydrophobic icroenvironment induced by the binding of chloroform at or near the ligand binding site. Chloroform severely quenched the intrinsic yptophan fluorescence of the protein and shifted the emission maxima towards blue region in all the albumins except PSA. However, ıantitative differences in both quenching and blue shift were noted in different serum albumins. This suggests that chloroform obably binds in the close vicinity of tryptophan residue(s) located in subdomain(s) IIA or IB and II both. The fluorescence of R-albumin complexes was also found to be sensitive to the presence of a small amount of chloroform. But the changes observed in e fluorescence of the bound pigment in presence of chloroform were less marked as compared to the changes in the intrinsic ıorescence of protein per se. Taken together, these results suggest that there is at least one conserved site for chloroform binding in l these albumins which is at or near the BR binding site. © 2000 Société française de biochimie et biologie moléculaire / Éditions ;ientifiques et médicales Elsevier SAS

ilirubin / serum albumins / chloroform / CD inversion / chirality / fluorescence quenching

. Introduction

Bilirubin (BR), a catabolic product of hemoglobin 'hen produced excessively in plasma, results in the evelopment of hyperbilirubinemia leading to a perma-ent neurologic disorder, kernicterus, in newborn infants 1]. Albumin acts as a biologic buffer against life threat-ning neurotoxic effects of BR which in a non-covalent ssociation transports it to the liver for conjugation and ubsequent excretion from the body [2, 3]. The study of ;R binding to albumin has been a long term discussion ecause of its use in developing various preventive ıeasures against fatal kernicterus in jaundiced infants [4]. 'arious serum albumins have shown differences in the inding of BR to high affinity site as characterized by different techniques including fluorescence and circular dichroism (CD) spectroscopy [5, 6]. Both spectral properties (CD and fluorescence) of BR-albumin complexes have been shown to be affected by change in the experimental conditions such as pH or presence of chloroform [6, 7]. The study of the effect of chloroform on the spectral properties of BR-albumin complex becomes important due to its use in the extraction of various BR isomers after photoirradiation of BR-albumin complex under in vitro conditions [8, 9] as well as its use in solubilizing BR in BR-albumin binding studies [6]. Non-specific association of chloroform with serum albumins and other proteins has been well documented [10]. The majority of proteins have been found relatively insensitive but for those which are sensitive, a trace amount of chloroform and other anesthetic molecules though has been shown to have little effect on secondary and tertiary structure but sufficient to perturb their normal functions [10–13]. This is evident from the chloroform dependent inversion of the characteristic bisignate CD spectrum of BR-HSA complex as a

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Effect of lysine modification on the conformation and indomethacin binding properties of human serum albumin

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Abstract

In order to study the involvement of lysine residues of human serum albumin (HSA) in the binding of indomethacin, HSA was treated with different molar excess of acetic anhydride, succinic anhydride and *O*-methylisourea which resulted in differently modified preparations: 30%, 62% and 87% acetylated, 20%, 34%, 64% and 78% succinylated and 21%, 43% and 86% guanidinated HSAs. All the preparations were found to be homogeneous with respect to charge as well as size as judged by polyacrylamide gel electrophoresis and gel filtration on a Seralose-6B column. Hydrodynamic and circular dichroic results showed that pronounced conformational changes (both tertiary and secondary structures) were induced in the maximally acetylated (87%) and succinylated (78%) preparations. On the other hand, guanidinated preparations showed no expansion in the hydrodynamic volume. The percent decrease in α-helical content was 34% for 87% acetylated, 31% for 78% succinylated and 10% for 86% guanidinated HSAs. A significant increase in the values of Stokes radii and frictional ratios (from 3.43 nm and 1.29 for native HSA to 4.07 nm and 1.52 for 87% acetylated and 4.35 nm and 1.60 for 78% succinylated HSAs, respectively) was also noticed in these highly modified preparations. Fluorescence quench titration results obtained at pH 7.4 and ionic strength 0.15 showed that only 54.1% and 64.7% binding of indomethacin at 4:1 drug/protein molar ratio was retained by 87% acetylated and 78% succinylated HSAs, respectively, as compared to 91% retention in binding in 86% guanidinated preparation. No reversal in the binding of drug to 87% acetylated and 78% succinylated HSA preparations was observed on increasing the ionic strength to 1.0. Therefore, it seems that one or two critical lysine residue(s) that can form salt linkage with the carboxyl group of indomethacin, was (were) probably modified in these preparations. A small decrease in the binding of drug to the guanidinated preparation also confirms the involvement of positive charge, probably contributed by lysine residue(s), in the binding of indomethacin to HSA.

Keywords: Acetylation; Guanidination; Human serum albumin; Indomethacin; Succinylation

1. Introduction

Human serum albumin (HSA) is the major transport protein of the mammalian blood circulation. Its ability to bind a broad spectrum of ligands, both exogenous and endogenous, seems to be correlated well with its structural flexibility to adopt its shape to the ligand [1]. Of particular pharmacological interest is the binding of drugs to serum albumin, most of which bind to this protein in a reversible manner. A detailed knowledge about the drug binding site is necessary to understand the distribution of any drug in the body and its competition with other drugs or with a natural catabolite, bilirubin [2], since the pharmacokinetic behaviour of drugs can be greatly dependent on the binding phenomenon [3]. Binding of indomethacin, a pharmacological agent used to promote constriction of the patent ductus arteriosus in premature infants, to HSA has been shown to be competitive in nature and utilizes both the bilirubin and diazepam binding functions of serum albumin [4–6]. No attempt regarding the involvement of various amino acid residues of HSA in the binding of indomethacin has yet been made to the best of our knowledge. On the other hand, involvement

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Partial characterization of goat brain proteins involved in bilirubin binding

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In order to characterize in terms of bilirubin binding, major proteins in the goat brain were separated into five different peaks, namely P_0, P_1, P_2, P_3 and P_4 on a Seralose-6B column (90.5 × 2.5 cm). The peak P_0 was eluted with the void volume of the column. The molecular weights and Stokes radii of the remaining peak proteins were: P_1 (1,06,727 and 4.11 nm), P_2 (59,256 and 3.25 nm), P_3 (18,713 and 1.71 nm) and P_4 (11,928 and 1.14 nm). Bilirubin binding studies indicated that three out of the five peak proteins, namely P_1, P_2, and P_3 showed bilirubin binding as characterized by the blue shift and hyperchromism in the visible absorption spectra and quenching of the protein fluorescence upon addition of bilirubin to these peak proteins.

HYPERBILIRUBINEMIA is an important pathological condition in the newborn and the possibility of low grade brain damage due to bilirubin toxicity is of interest to clinicians[1]. Decreased albumin binding capacity, increased bilirubin concentration or low albumin levels, can account for increased bilirubin deposition in the brain thus leading to the development of the clinical syndrome, kernicterus which may result in infant death[2]. Bilirubin neurotoxicity may be mediated by a number of mechanisms due to its increased permeability in neuronal membranes. Recently, it was reported that the high neonatal serum bilirubin levels adversely affected hearing[3], while in newborn piglets its prolonged infusion modified the N-methyl-D-aspartate (NMDA) receptor/ion channel complex in the cerebral cortex[4]. In spite of extensive research, the mechanisms of bilirubin toxicity in the brain and characterization of the bilirubin binding proteins remain elusive. In this paper, we report our data on the characterization of some bilirubin binding proteins in goat brain.

Fresh goat brain, obtained from a local slaughterhouse, was dissected and made free of membranous tissues and circulatory debris. Then, it was cut into small pieces and homogenized in a Remi mixer for about 5 min with three volumes of 0.06 M sodium phosphate buffer, pH 7.0. The crude homogenate was centrifuged at 6000 g for 30 min. The supernatant was filtered through millipore filter (0.25 mm) and the residue was discarded. It was then applied directly on a Seralose-6B column (90.5 × 2.5 cm). The different peak fractions, obtained

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Visualization of serum albumin on electrophoretic gels using the specific ligand bilirubin

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Abstract

Serum albumin, when incubated with bilirubin prior to electrophoresis, was visualized as a yellow-colored band during the electrophoretic run and did not require any staining. Furthermore, free bilirubin served as a tracking dye. A minimum of 20 μg of protein was detected very well by this method. The formation of a bilirubin–albumin complex did not affect the electrophoretic mobility as the protein complexed with bilirubin as well as free albumin moved with the same mobility. Only a single protein band was visualized by this method after electrophoresis of human plasma. The method is simple, less time-consuming and may be used in identifying bilirubin-binding proteins in various biological samples.

Keywords: Bilirubin; Electrophoresis; Human serum albumin

1. Introduction

Bilirubin, a catabolic product of hemoglobin, is toxic in its free form to various cell types [1,2]. Under normal physiological conditions, the toxicity of this substance is checked by albumin, which carries it to the liver for conjugation with glucuronic acid and excretion [3]. However, when the production of bilirubin exceeds the binding

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